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NEW DELHI - 110 008.

IN/04/203

I, the undersigned being an officer duly authorized in accordance with the provision of the Patent Act, 1970 hereby certify that annexed hereto is the true copy of the ***Application, Provisional & Complete Specification*** *and* ***Drawing Sheets*** *filed in connection with Application for Patent No.* ***882/Del/2003*** *dated* ***9th July 2003.***

Witness my hand this 1st day of September 2004.

(S.K. PANGASA)
Assistant Controller of Patents & Designs

0882 DEL 03

FORM 1

THE PATENTS ACT, 1970
(39 of 1970)
APPLICATION FOR GRANT OF A PATENT
(See sections 5(2), 7, 54 and 135 and rule 33A)

09 JUL 2003

1. I/We, **INDIAN COUNCIL OF MEDICAL RESEARCH,** an Indian registered body incorporated under the Registration of Societies Act (Act XXI of 1860), and having their registered office at V. Ramalingaswami Bhawan, Ansari Nagar, Post Box 4911, New Delhi 110 029, India

2. hereby declare -

(a) that I am/we are in possession of an invention titled:

: **"TYROSINE PHOSPHATASES OF MYCOBACTERIUM TUBERCULOSIS AS POTENTIAL TARGETS FOR DEVELOPING ANTITUBERCULAR DRUGS."**

(b) that the **Provisional specification** relating to this invention is filed with this application.

(c) that there is no lawful ground of objection to the grant of a patent to me/us.

3. Further declare that the inventor(s) for the said invention is / are :

(a) **Anil Kumar Tyagi,** of Department of Biochemistry, University of Delhi south Campus, Benito Juarez Road, New Delhi 110021, India,

(b) **Ramandeep Singh,** of Department of Biochemistry, University of Delhi south Campus, Benito Juarez Road, New Delhi 110021, India,

(c) **Vivek Rao,** of Department of Biochemistry, University of Delhi south Campus, Benito Juarez Road, New Delhi 110021, India,

(d) **Vadakkuppattu Devasenapathi Ramanathan,** of Tuberculosis Research Centre, Mayor V. R. Ramanathan Road, Chetput, Chennai, Tamil Nadu, India,

(e) **Chinnambedu Nainarappan Paramasivan,** of Tuberculosis Research Centre, Mayor V. R. Ramanathan Road, Chetput, Chennai, Tamil Nadu, India,

(f) **Paranji Ramaiyengar Narayanan,** of Tuberculosis Research Centre, Mayor V. R. Ramanathan Road, Chetput, Chennai, Tamil Nadu, India, and

(g) **Yogendra Singh,** of Institute of Genomics and Integrative Biology, Mall Road, Delhi – 110 007

All the above Inventors are Indian citizens.

4. I/We, claim the priority from the application(s) filed in convention countries, particulars of which are as follows: NIL.
and declare that above application or each of the above applications was the first application(s) in a convention country / countries in respect of my / our invention.

5. I/We, state that the said invention is an improvement in or modification of the invention, the particulars of which are as follows and of which I/We are the applicant/patentee: NA

6. I/We, state that the application is divided out of my/our application, the particulars of which are given below: NA
and pray that this application deemed to have been filed on
under Section 16 of the Act.

7. That I am/We are the assignee or legal representative of the true and first inventors.

8. That my/our address for service in India is as follows:

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9. Following declaration was given by the inventor(s) or applicant(s) in the convention country:

I/We the true and first inventors for this invention of or the applicant(s) in the convention country declare that the applicant(s) herein is/are my/our assignee or legal representative.

Anil Kumar Tyagi _____

Ramandeep Singh _____

Vivek Rao _____

Vadakkuppattu Devasenapathi Ramanathan _____

Chinnambedu Nainarappan Paramasivan _____

Paranji Ramaiyengar Narayanan _____

Yogendra Singh _____

10. That to the best of my/our knowledge, information and belief the fact and matters stated herein are correct and that there is no lawful ground of objection to the grant of patent to me/us on this application.

11. Following are the attachment with the application:

(a) Provisional specification, claims, drawings and abstract in triplicate
(b) Form 3
(c) Form 5
(d) Official fee Rs.3000/-

I/We request that a patent may be granted to me/us for the said invention.

Dated this 9th day of July, 2003.

Rajeshwari

RAJESHWARI H
OF K & S PARTNERS
ATTORNEY FOR THE APPLICANTS

To
The Controller of Patents
Delhi

FORM 2 0382/DEL/03

THE PATENTS ACT, 1970
(39 of 1970)

09 JUL 2003

PROVISIONAL SPECIFICATION
(See section 10)

"TYROSINE PHOSPHATASES OF MYCOBACTERIUM TUBERCULOSIS AS POTENTIAL TARGETS FOR DEVELOPING ANTITUBERCULAR DRUGS."

PUBLICATION

INDIAN COUNCIL OF MEDICAL RESEARCH, an Indian registered body incorporated under the Registration of Societies Act (Act XXI of 1860), and having their registered office at V. Ramalingaswami Bhawan, Ansari Nagar, Post Box 4911, New Delhi 110 029, India

The following specification describes the nature of the invention.

TYROSINE PHOSPHATASES OF *MYCOBACTERIUM TUBERCULOSIS* AS POTENTIAL TARGETS FOR DEVELOPING ANTITUBERCULAR DRUGS.

Field of the invention:

The invention demonstrates essential role of tyrosine phosphatases (MptpA and MptpB) in the pathogenesis of *M. tuberculosis*. These secretory tyrosine phosphatases represent attractive targets for the development of new anti-tubercular drugs for short-term therapy against tuberculosis.

Background of the invention:

One-third of world's population is latently infected with *M. tuberculosis*. Eight million new cases of active diseases develop each year & three million people succumb to this disease every year (Dye *et al.*, 1999). With the advent of HIV & emergence of multidrug resistant strains of *M. tuberculosis*, the problem has increased manifold (Horsburgh, 1991; Barnes *et al.*, 1991 and Bloch *et al.*, 1994). The current treatment of disease usually involves combination chemotherapy based on isoniazid, pyrazinamide, rifampicin & ethambutol. In general, 6 months long course is required for effective treatment, which often results in poor compliance on the part of patients, who stop drug intake as soon as they begin to feel better. This leads to development of drug resistant forms of bacilli, which are able to survive routine drug therapy. Multidrug resistant tuberculosis (MDR-TB) is defined as a disease due to tubercle bacilli resistant to at least isoniazid and rifampicin, the two most powerful anti-tubercular drugs. Such a precarious scenario demands development of new drugs that can act on new targets and can be effective in relatively shorter periods so that the patients do not develop resistance to these drugs. The present invention can lead to the development of such target specific anti-tubercular drugs useful for short-term therapies.

Sequence analysis of various prokaryotes has shown the presence of eukaryotic like serine/threonine and tyrosine phosphatases in bacterial pathogens. In various pathogenic bacteria like *Yersinia pseudotuberculosis*, *Salmonella typhimurium* and enteropathogenic *E.coli* tyrosine phosphatases have been shown to act as major virulence determinants (Guan and Dixon., 1990; Galyov *et al.*, 1993 and Kaniga *et al.*, 1996)

YopH, one of the PTPases, is encoded by the yersiniae virulence plasmid and has been identified as an essential virulence factor (Bliska *et al.*, 1991). YopH comprises of several domains including amino terminal sequences involved in secretion, translocation and chaperone binding; a central proline rich SH3 – binding domain and a carboxyl terminal catalytic domain that is homologous to a domain in the eukaryotic PTPases (Sory *et al.*, 1995). It is postulated that YopH disrupts a general phagocytic mechanism as both Fc receptor and complement mediated phagocytosis is inhibited by YopH. (Ruckdeschel *et al.*, 1996 and Fallman *et al.*, 1995). Two of the YopH substrates, $p130^{cas}$ and paxillin are proteins involved in connecting integrins to the actin cytoskeleton and the third one is a tyrosine kinase (Persson *et al.*, 1997 and Black *et al.*, 1997). The possible explanation for the role of YopH protein is that it inhibits uptake of bacteria mediated by the interaction of the bacterial outer membrane protein invasin with cellular β1 integrin. According to this model, invasin binding stimulates tyrosine phosphorylation of cellular targets, leading to cytoskeletal rearrangements and bacterial uptake. YopH dephosphorylates the protein required for this activity. Recent studies have shown that YopH also inhibits Akt pathway and phosphatidylinositol 3 – kinase dependent secretion of interleukin 2 in macrophages (Sauvonnet *et al.*, 2002).

S. typhimurium encodes a tyrosine phosphatase, SptP comprised of modular domains. The amino-terminus of SptP exhibits sequence homology to the Exotoxin S from *P. aeruginosa* and YopE from *Yersinia* spp.. Exotoxin S is an ADP ribosyl transferase that has been implicated in *P. aeruginosa* in the induction of host cell injury and is known to be a virulence factor of *P. aeruginosa*. The carboxyl terminus of SptP showed homology to the eukaryotic like protein tyrosine phosphatases. The carboxyl terminus of SptP protein is homologous to YopH and the catalytic domain of the eukaryotic PTPase. The cysteine residue at position 481 is essential for its catalytic activity as mutation of this conserved cysteine residue abolishes the phosphatase activity (Kaniga *et al.*, 1996). Kaniga *et al* showed that *sptP* mutants are defective in the colonization of spleens of orally infected BALB/c mice. SptP has been shown to possess an *in vitro* GTPase activating protein (GAP) activity towards two host GTP binding proteins, Rac-1 and Cdc42 that play an important role in the cytoskeletal dynamics (Fu and Galan, 1999). It has been suggested that the GAP activity of SptP could down regulate signaling through Cdc42 and Rac that could rebuild the actin cytoskeleton after Salmonella entry. Fu and Galan have shown that

microinjection of purified GST-SptP into cultured cells results in the disruption of actin cytoskeleton and the disappearance of stress fibers (Fu and Galan, 1999).

Prior art:

Allelic exchange by homologous recombination is a powerful tool to study gene functions, identification of virulence factors and development of auxotrophic mutants. "Gene knockout" technique involves the replacement of a wild type gene with it's non-functional counterpart. Such targeted mutations are widely used to study gene functions in mammalian, eukaryotic and bacterial cells (Guilhot *et al.*, 1992; Myers *et al.*, 1994; Reyrat *et al.*, 1995; Baulard *et al.*, 1996; Balsubramaninan *et al.*, 1996;; Azad *et al.*, 1996 ; Azad *et al.*, 1997; Hinds *et al.*, 1999; parish *et al.*, 1999;; Pelicic *et al.*, 1997; Bardarov *et al.*, 1997 and Raynaud *et al.*, 2002).

Sequence analysis of *M. tuberculosis* genome revealed the presence of 11 serine/threonine kinases and two tyrosine phosphatases (Cole *et al.*, 1998). Both genes having sequence homology with known tyrosine phosphatases were PCR amplified by using gene specific primers and *M. tuberculosis* genomic DNA, cloned in a prokaryotic expression vector, pGEX5x-3 and purified from *E. coli* as GST fusion proteins (Koul *et al.*, 2000). The GST fusion proteins were able to dephosphorylate the phospho-tyrosine residue of myelin basic protein but were unable to dephosphorylate phospho-serine and phospho-threonine residues of myelin basic protein. Site directed mutagenesis of cysteine residues in the catalytic motif (Cys11 in the case of MptpA and Cys160 in the case of MptpB) abolished the enzymatic activity (Koul *et al.*, 2000). By Southern blot analysis, it was revealed that *mptpA* is present in fast growing as well as slow growing species of mycobacteria. However, while the mptpB was present in slow growers it was found to be absent in *M. smegmatis*, a fast growing species. (Koul *et al.*, 2000). In view of the known role of tyrosine phosphatases in the pathogenesis of several bacterial pathogens, characterization of the role of MptpA and MptpB in the pathogenesis of *M. tuberculosis* would be an important step towards the development of anti-tubercular drugs.

Objects of the invention:

The main objective of the present invention is to identify mycobacterial tyrosine phosphatases (MptpA and MptpB) as potential drug targets for the development of new anti-tubercular drugs.

Another object of the present invention is to clone *mptpA* along with it's left and right flanking regions in a non – replicative suicidal vector.

Another object of the invention is to disrupt *mptpA* in the genome of *M. tuberculosis* by homologous recombination using alkali pretreated non – replicative vector.

Another object of the present invention is to demonstrate the impaired ability of the *mptpA* mutant strain in comparison to the parental strain of *M. tuberculosis* to survive in the activated macrophages.

Another object of the present invention is to demonstrate the impaired ability of the *mptpA* mutant strain as compared to the parental strain of *M. tuberculosis* to survive in guinea pigs.

Another object of the present invention is to clone *mptpB* along with it's left and right flanking regions in a non – replicative suicidal vector.

Another object of the invention is to disrupt *mptpB* in the genome of *M. tuberculosis* by homologous recombination using alkali pretreated non – replicative vector.

Another object of the present invention is to demonstrate the impaired ability of the *mptpB* mutant strain in comparison to the parental strain of *M. tuberculosis* to survive in the activated macrophages.

Another object of the present invention is to demonstrate the impaired ability of the *mptpB* mutant strain as compared to the parental strain of *M. tuberculosis* to survive in guinea pigs.

Another object of the present invention is to demonstrate that tyrosine phosphatases (MptpA and MptpB) are essential for the pathogenesis of *M. tuberculosis*.

Summary of the invention:

The present invention relates to identification of the role of Protein tyrosine phosphatases (MptpA and MptpB) in the pathogenesis of *Mycobacterium tuberculosis* and use of these two proteins as targets for the development of anti tubercular drugs.

Detailed description of the invention:

The present invention relates to identification of mycobacterial protein tyrosine phosphatases (MptpA and MptpB) as potential targets for developing new anti tubercular drugs:

1. The present invention provides a method for demonstration of the role of MptpA in the pathogenesis of *M. tuberculosis.* This method provides for:

   a) Extraction of the genomic DNA from *M. tuberculosis* followed by PCR amplification of *mptpA* along with its left and right flanking regions using gene specific primers of sequence IDs 1 to 4 as below:

   Sequence ID1: CCA TCA TGA CTG TGG AAC CTA TTC CTG TCG GCC

   Sequence ID2: GGG CAT ATG GGC TCC ATT CGC CGG ACT CGC CG

   Sequence ID3: GGG CAT ATG GGC TGG ATT CGC CGG ACT CGC CG

   Sequence ID4: CCA TCA TGA GTC GGT TAC CCC CGT ATA GCC CGG

   b) Sequencing of the PCR amplicons by automated sequencing, construction of the targeting vector pAKΔA.

   c) Disruption of *mptpA* in the genome of *M. tuberculosis* by using alkali pretreated pAKΔA by homologous recombination, resulting in the *mptpA* mutant strain of *M. tuberculosis.*

   d) Comparative analysis of survival of the *mptpA* mutant and the parental strain of *M. tuberculosis* in the activated mouse macrophages (J774A.1).

   e) PCR amplification of complete open reading frame of *mptpA* using gene specific primers of sequence IDs 5 to 6 and *M. tuberculosis* genomic DNA.

   Sequence ID5: G CAT ATG CAT CTG TGA TCC GCT GCA CGT CAC ATT C

   Sequence ID6: CCG AGG CGT TCA ACT CGG TCC GTT CCG CGC GCG AC

   f) Sequencing of the PCR amplicon by automated sequencing and construction of pSD5 60-*mptpA* for reintroduction of MptpA into the *mptpA* mutant strain of *M. tuberculosis* resulting in the complemented strain.

g) Challenging the guinea pigs of sound health subcutaneously with the parental, *mptpA* mutant strain from step 1c) and complemented strain of *M. tuberculosis* from step 1f).

h) Comparative analysis of *in vivo* survival of the parental, *mptpA* mutant and complemented strain of *M. tuberculosis* in guinea pigs.

2. The present invention provides a method for demonstration of the role of MptpB in the pathogenesis of *M. tuberculosis*. This method provides for:

a) Extracting the genomic DNA from *M. tuberculosis* followed by PCR amplification of *mptpB* along with its left and right flanking regions using gene specific primers of sequence IDs 7 to 10 as below:

Sequence ID7: CCA TCA TGA CGT CGT CTGACA AC GAG CGT CC

Sequence ID8: GGG CAT ATG GCA ACA CCC CGG CCG CCC GCT CG

Sequence ID9: GGG CAT ATG ACG CTC GGC TGT TTG CGG CAG CTC G

Sequence ID10: CCA TCA TGA CGG TGG GTC GCC CCG CGG TGC GG

b) Sequencing of the PCR amplicons by automated sequencing and construction of the targeting vector pBKΔB.

c) Disruption of *mptpB* in the genome of *M. tuberculosis* by using U. V. irradiated pBKΔB by homologous recombination, resulting in the *mptpB* mutant strain of *M. tuberculosis*.

d) Comparative analysis of survival of the *mptpB* mutant and the parental strain of *M. tuberculosis* in the activated mouse macrophages (J774A.1).

e) PCR amplification of complete open reading frame of *mptpB* using gene specific primers of sequence IDs 11 to 12 and *M. tuberculosis* genomic DNA.

Sequence ID11: CG CAT ATG CGA TGG CTG TCC GTG AAC TGC CGG G

Sequence ID12: CAC GCG TTC CTG CGA GCA GCA CCC CGC GCA TCC G

f) Sequencing of the PCR amplicon using automated sequencer and construction of pSD5 60-*mptpB* for reintroduction of MptpB in the *mptpB* mutant strain of *M. tuberculosis* resulting in the complemented strain.

g) Challenging the guinea pigs of sound health with the parental, *mptpB* mutant strain from step 2c) and complemented strain of *M. tuberculosis* from step 2f).

h) Comparative analysis of *in vivo* survival of the parental, *mptpB* mutant and complemented strain of *M. tuberculosis* in guinea pigs.

The mutant strain lacking tyrosine phosphatases associated with either MptpA or MptpB was employed to understand the role of these proteins in the survival of *M. tuberculosis* in murine macrophages and in the ability of the mutants to cause disease in guinea pigs. Both the parental and the *mptpA* mutant strains differed in their ability to survive in the activated macrophages. In activated macrophages approximately 45%, 50% and 70% killing of the wild type bacilli was observed, at days 2,4 and 6 post infection, respectively, in comparison to 70%, 95% and 98% killing of the *mptpA* mutant strain at days 2, 4 and 6 post-infection, respectively. Consistent with these observations, disruption of *mptpA* impaired the ability of *M. tuberculosis* to survive in lungs and spleen of infected guinea pigs. At 3 weeks post-infection the bacillary load in the spleens and lungs of animals infected with the *mptpA* mutant strain was approximately ~1log less in comparison to the bacillary load in the spleens and lungs of animals infected with the parental strain. However, at six weeks post-infection the difference in bacillary load increased from 10-folds to 90-folds. An approximately 90-fold (1.9 log) reduction in the bacillary load in the spleens and lungs of animals infected with the *mptpA* mutant strain in comparison to the parental strain was found to be statistically significant ($p<0.05$).

Both the parental and the *mptpB* mutant strains differed in their ability to survive in the activated macrophages. An approximately 5-fold reduction in the number of intracellular *mptpB* mutant (10%) was observed in comparison to the number of intracellular parental strain (50%) at 4 days post-infection. At 6 days post-infection, approximately 7 fold reduction was observed in the number of intracellular *mptpB* mutant strain (4%) in comparison to the number of internalized parental strain (28.6%) suggesting that the *mptpB* mutant strain was more sensitive to killing as compared to the wild type strain by activated macrophages. By using the guinea pig model of infection we showed a significant reduction in the ability of the mutant strain to survive in the host. Initially, at 3 weeks post-infection, no difference was observed in the splenic bacillary load of the animals infected with either the parental or the *mptpB* mutant. However, an approximately 70-fold (1.7 log) reduction in bacillary load was observed in the spleen of the animals infected with the *mptpB* mutant strain as compared to the bacillary load from the animals infected with the wild type strain at 6 week post-infection **($p<0.05$)**. That the loss of virulence of *M. tuberculosis* was a

direct consequence of disruption of *mptpB*, was shown by the ability of the complemented strain to establish an infection and survive in the host tissues at levels comparable to those observed in case of the wild type *M. tuberculosis*.

Phosphatases and kinases have been identified as virulence determinants in bacterial pathogens. Protein tyrosine phosphatases YopH and SptP from *Yersinia pseudotuberculosis* and *Salmonella typhimurium*, respectively, have been well characterized. Protein tyrosine dephosphorylation of macrophage proteins by YopH prevents phagocytosis-linked signaling pathways (Bliska *et al.*, 1991; Bliska and Black, 1995; Fallman *et al.*, 1995; Andersson *et al.*, 1996 and Black and Bliska 1997). Similarly, SptP of *S. typhimurium* has been shown to play an important role in cytoskeletal rearrangements by interacting with Rac-1 and Cdc42 leading to internalization of bacteria into non-phagocytic cells (Fu and Galan, 1999). Since phosphorylation and dephosphorylation regulate several crucial processes in eukaryotic cells, secreted tyrosine phosphatases may serve as key molecules that modify host proteins to its advantage and enable tubercle bacilli to survive within the host. In order to evaluate the role of MptpA and MptpB in the pathogenesis of *M. tuberculosis*, *mptpA* and *mptpB* mutant strains were constructed by homologous recombination. Using the guinea pig model of experimental tuberculosis, we report that mutant strains showed impaired ability to survive in guinea pigs.

(A) Disruption of *mptpA* in *M. tuberculosis* and its effect on the pathogenesis of *M. tuberculosis*.

In order to evaluate the role of MptpA in the pathogenesis of *M. tuberculosis*, an *mptpA* mutant strain was constructed by using a non-replicative suicidal vector pAKΔA. The targeting vector, pAKΔA carried the coding region of *mptpA* along with it's 1135 bp upstream and 1240 bp downstream flanking sequences of *mptpA*. A portion of the coding region (112 bp) of MptpA was deleted and replaced with gene conferring resistance to hygromycin in pAKΔA. Electroporation of *M. tuberculosis* Erdman with non-replicative vector, pAKΔA and alkali denatured pAKΔA resulted in 39 and 2 hygromycin resistant transformants, respectively on 7H10 plates supplemented with hygromycin (50μg/ml). All the transformants were PCR positive for hygromycin resistance gene suggesting that plasmid borne *mptpΔA::hyg*^{r} had integrated into the mycobacterial genome. Allelic exchange by homologous recombination should result in incorporation of the hygromycin resistance gene but not the vector backbone (carrying kanamycin resistance gene) into the mycobacterial genome. Thus, the transformants were screened for kanamycin resistance gene by

PCR using gene specific primers. The transformants obtained upon electroporation of pAKΔA were PCR positive for the kanamycin cassette, where as the two transformants obtained upon electroporation of alkali denatured pAKΔA were PCR-negative for the kanamycin cassette. These results indicated that homologous recombination at *mptpA* locus had occurred in the case of transformants obtained upon electroporation of alkali denatured DNA. Thus, transformants resistant to hygromycin but sensitive to kanamycin were selected to score for homologous recombination event.

The disruption of *mptpA* in the mycobacterial genome was verified by Southern blot analysis using *mptpA* specific DNA probe (492 bp DNA fragment containing the entire coding region of MptpA). As expected, for allelic exchange event to occur at homologous site, in the lanes corresponding to the two hyg^r kan^s transformants, a single hybridizing fragment 4.1 kb, 2kb longer than that in the wild type strain (2.1kb) was observed. This increase in the size of the band by 2.0 kb in both hyg^r kan^s transformants corresponded to the replacement of 112bp internal fragment of *mptpA* with hygromycin resistance gene (Fig. 1A). Immunoblot analysis of whole cell lysate demonstrated that disruption of *mptpA* resulted in lack of expression of MptpA in the mutant strain (Fig. 1B), which could be restored in the mutant strain by electroporation of pSD5-*mptpA*.

To investigate the role of MptpA in the intracellular survival of *M. tuberculosis*, the survival rates of *mptpA* mutant and its parental strain were compared in resting as well as in IFN-γ activated mouse macrophage cell line, J774A.1. The numbers of intracellular surviving bacteria were calculated at days 0,2,4,6 and 8 post-infection. Both parental as well as *mptpA* mutant strain displayed a similar pattern of intracellular growth in resting macrophages. While at the initial time point (day0) bacillary counts were approximately 2×10^4 per well. The bacillary load increased at later time points attaining peak values of 2×10^5 at day 8 post-infection. These results showed that both parental as well as *mptpA* mutant strains of *M. tuberculosis* exhibited comparable capacity of infection and multiplication in resting macrophages (Fig. 2A). However, both the strains differed in their ability to survive in IFN-γ activated macrophages. In activated macrophages approximately 45%, 50% and 70% killing of wild type bacilli was observed, at days 2,4 and 6 post-infection, respectively, in comparison to 70%, 95% and 98% killing of *mptpA* mutant strain at days 2, 4 and 6 post-infection, respectively (Fig. 2B). These observations indicated

that disruption of *mptpA* had impaired the ability of *M. tuberculosis* to survive in IFN-γ activated macrophages.

To determine whether MptpA plays a role in the pathogenesis of *M. tuberculosis*, guinea pigs in groups of 16 animals were infected subcutaneously with 5×10^7 cfu of parental, mutant or complemented strain of *M. tuberculosis*. Animals were euthanised 3 weeks and 6 weeks post-infection. At both time points of euthanisation (7 animals per group), number of colony forming units in spleen and lungs were enumerated (represented as $\log_{10}$cfu for each group).

The *mptpA* mutant strain was significantly attenuated for growth in guinea pig model of tuberculosis. At 3 weeks post-infection a 9-fold reduction was observed in the bacillary load in spleens of animals infected with *mptpA* mutant strain ($\log_{10}$ 5.09 ± 0.23) as compared to the parental strain ($\log_{10}$ 5.99 ± .27, Fig. 3A). A similar reduction in cfu was also observed in the lungs of animals infected with *mptpA* mutant strain, ($\log_{10}$ 3.07 ± .13) as compared to ($\log_{10}$ 3.95 ± 0.32) in the lungs of animals infected with the parental strain (Fig. 4A). The differences in the bacterial load in the spleen and lungs of animals infected with *mptpA* mutant strain as compared to the bacterial load of animals infected with parental strain increased from 9 folds to 90 folds at six weeks post-infection. The bacillary load in the animals infected with *mptpA* mutant strain was $\log_{10}$ 4.83 ± 0.43 for spleens and 3.71± 0.30 for lungs, when compared to the bacillary load in animals infected with parental strain 6.73 ± 0.33 for spleens and 5.62 ± 0.38 for lungs (Fig. 3B and 4B, respectively). The reduction in the bacillary load in the spleens and lungs of animals infected with *mptpA* mutant strain was found to be statistically significant ($p < 0.002$ in the case of spleens and $p < 0.001$ in the case of lung, respectively). Complementation of *mptpA* mutant strain with a functional copy of MptpA partially restored the virulence of mutant strain (Fig 3 and 4).

Sections of liver and lung from various groups were analysed histologically to determine the extent of tissue damage. Fig. 5 depicts the mean percentage of granuloma and cellular composition in liver granuloma of animals at 3 weeks post-infection. At 3 weeks post-infection, the animals infected with the parental strain exhibited 5.4% liver granuloma. The liver granuloma comprised of 10% lymphocytes, 8% macrophages and 82% epitheloid cells. In case of animals infected with the *mptpA* mutant strain, 10% liver granuloma was observed and the granuloma

comprised of 21% lymphocytes, 11% macrophages and 68% epitheloid cells. 4.6% liver granuloma was observed in case of animals infected with the complemented strain and the granuloma comprised of 30% lymphocytes, 6% macrophages and 64% epitheloid cells (Fig. 5).

In case of lung, no significant difference was observed in the percentage of granulomatous tissue and cellular composition of the granuloma in case of animals infected with various strains. The animals infected with parental strain exhibited 14% lung granuloma and lung granuloma comprised of 30% lymphocytes and 70% macrophages. In case of animals infected with *mptpA* mutant strain 21.5% lung granuloma was observed and the granuloma comprised of 30% lymphocytes and 70% macrophages. The animals infected with complemented strain exhibited 20% granuloma and granuloma comprised of 24% lymphocytes and 56% macrophages. Representative sections of liver and lung of animals infected with the parental, or *mptpA* mutant strain or complemented strain at 3 weeks post-infection are shown in Fig. 6.

(B) Disruption of *mptpB* in *M. tuberculosis* and its effect on the pathogenesis of *M. tuberculosis*:

In order to establish whether MptpB plays a role in the pathogenesis of *M. tuberculosis*, a *mptpB* mutant strain of *M. tuberculosis* was constructed by using a non-replicative suicidal vector pBKΔB. The targeting vector, pBKΔB carried the coding region of *mptpB* along with 1045 bp upstream and 1140 bp downstream flanking sequences. A portion of the coding region (108 bp) of MptpB was deleted and replaced with the gene conferring resistance to hygromycin in pBKΔB. The vector also carried the gene conferring resistance to kanamycin in its backbone as a second antibiotic selection marker for negative screening of allelic exchange events at the homologous site.

Electroporation of *M. tuberculosis* with pBKΔB and U.V. irradiated pBKΔB resulted in 22 and 3 hygromycin resistant transformants, respectively. PCR analysis revealed that all the transformants contained hygromycin cassette indicating that these colonies were not spontaneous resistance mutants and arose from integration of the suicidal vector into the mycobacterial genome. Allelic exchange event by homologous recombination should result in the incorporation of hygromycin resistance gene but not the vector backbone (having kanamycin resistance gene) into the mycobacterial genome. Thus, transformants resistant to hygromycin but

sensitive to kanamycin were selected to screen for homologous recombination event. All the transformants obtained on electroporation of untreated DNA were kanamycin resistant while the three transformants obtained on electroporation of U.V. pretreated DNA were sensitive to kanamycin. This suggested that an allelic exchange event at the homologous site had taken place in the case of these three hyg^r kan^s transformants obtained upon electroporation of U.V. irradiated DNA.

mptpB gene disruption was assessed by hybridization analysis of genomic DNA isolated from the parental *M. tuberculosis* strain and three hyg^Rkan^s transformants. A DNA fragment containing the entire coding region (831 bp) of *mptpB* was used as probe. Southern blot analysis showed presence of a 1.85 kb band in the parental strain whereas a 3.8 kb band was observed in all the three hyg^Rkan^s transformants as expected upon replacement of 108 bp internal fragment of *mptpB* with hygromycin resistance gene cassette (Fig. 7A). These results indicated that *mptpB* was disrupted in all the three hyg^Rkan^s transformants. Expression of MptpB was analysed in the mutant strains using polyclonal sera raised against MptpB in rabbit. Western blot analysis showed absence of MptpB expression in all the three mutant strains (Fig. 7B). The complemented strain was constructed by electroporation of pSD5-*mptpB* into electrocompetent cells of the mutant strain. The electroporation of pSD5-*mptpB* restored the expression of MptpB in the complemented strain (Fig. 7B).

To study the effect of disruption of *mptpB* gene on the intracellular survival of *M. tuberculosis*, resting and IFN-γ activated murine macrophage cells were infected with either the wild type or *mptpB* mutant strain of *M. tuberculosis*. The number of surviving intracellular bacteria was determined on days 0, 2, 4, 6 and 8 post - infection. Both parental as well as the *mptpB* mutant strain displayed a similar pattern of intracellular growth at all time points of study (Fig. 8A). While at the initial time point (day 0) the bacillary counts were approximately 10^4 cfu/well, the bacillary load increased at later time points attaining the peak values of ~ 10^5 cfu at 8 days post - infection. These results showed that both parental as well as the *mptpB* mutant strain exhibited comparable capacity of infection and multiplication in resting mouse macrophages. However, the two strains showed differences in their ability to survive in the activated macrophages. The number of wild type *M. tuberculosis* and *mptpB* mutant was maximum and comparable at the initial time point (~10^4 cfu/well, at day 0). At later time points, a reduction in the number of bacilli was observed in both cases. While the wild type *M. tuberculosis* was reduced to 50% and 28.6% at days 4 and 6 post- infection, respectively, a much sharper decline was noted in the

case of *mptpB* mutant which was reduced to 10% and 4% at days 4 and 6 post – infection, respectively (Fig. 8B). These observations indicated that disruption of *mptpB* gene had impaired the ability of *M. tuberculosis* to survive in IFN-γ activated macrophages.

To determine whether the disruption of *mptpB* gene would have any effect on the survival of *M. tuberculosis in vivo*, guinea pigs in groups of eight animals were infected subcutaneously with $5x10^5$ cfu of either parental, mutant or the complemented strain of *M. tuberculosis*. Animals were euthanized three weeks and six weeks post-infection. At both time points of euthanization, spleens were homogenized and viable bacilli were enumerated (represented as $\log_{10}$ cfu for each group).

It was observed that at 3 weeks post-infection, the mean total score of the animals infected with mutant strain was 26, which was comparable to the scores in case of animals infected with parental (28) and complemented strain (30, Fig.9A). These results were commensurate with the splenic cfu obtained for various groups on euthanization of animals at 3 weeks post-infection. The bacterial load in the spleen of animals infected with the mutant strain was $\log_{10}$ 3.71, which was comparable to the bacterial load in the spleens of animals infected with parental ($\log_{10}$ 3.73) and complemented strain ($\log_{10}$ 3.68, Fig.10A). However, the total scores of the animals infected with mutant strain at the end of six weeks was significantly lower (12) than the total score of animals infected with parental (35, $p<0.02$) and complemented strain (33, $p<0.02$, Fig.9B). The animals infected with mutant strain exhibited a significant reduction of bacillary load in spleen ($\log_{10}$ 3.07) when compared to bacillary load in spleen of animals infected with parental ($\log_{10}$ 4.77, $p<0.002$) and complemented strain ($\log_{10}$ 4.45, $p<0.003$, Fig. 10B). Thus, an approximately 3-fold reduction in total score and a 50 to 70-fold reduction in the bacillary load in spleens was observed in animals infected with *mptpB* mutant strain in comparison to parental or complemented strains.

Sections of liver and lung from animals in various groups were subjected to histological analysis to determine morphology of the organs, the presence and extent of granuloma and the type and number of infiltrating cells. It was observed that at three weeks there were no significant histological differences in liver and lung of animals infected with either parental, or mutant or complemented strain. At 3 weeks post-infection animals from all 3 groups showed no difference in the extent or composition of granuloma. In case of liver, granuloma consisted mainly of epitheloid

cells and lymphocytes, while the lung granuloma comprised mainly of lymphocytes macrophages and a few epitheloid cells (Fig. 11A and 11B). At six weeks post – infection, in the case of animals infected with wild type and complemented strain, the liver sections showed multiple well-defined granuloma comprising of epitheloid cells and lymphocytes. However, the liver tissue from animals infected with the *mptp*B mutant strain exhibited a distinct qualitative difference with respect to the presence of epitheloid cells with only a few lymphocytes. In case of lung tissues, the animals infected with the wild type and complemented strain showed extensive granulomas comprising of lymphocytes and macrophages. In contrast, the lung tissue from animals infected with the mutant strain showed partly organized granuloma mainly of lymphocytes (Fig. 12A and 12B).

Statistical analysis

Data are depicted as arithmetic mean ± standard error mean. Data were analyzed for statistical significance using the Student's t test. Differences between the guinea pig groups were considered significant if p values were <0.05.

Brief description of drawings:

Figure 1:

(A) Southern Blot analysis of the wild type (WT) and *mptpA* mutant (MT1 and MT2) strains of *M. tuberculosis*.

Genomic DNAs (3μg) from the wild type (WT) and *mptpA* mutant strain (MT1 and MT2) of *M. tuberculosis* were digested with *Not* I, separated on 1.2% agarose gel, transferred to Hybond N membrane and probed with ^{32}P labeled *mptpA* DNA fragment. The size of the DNA standards are shown on the left side of the gel and the size of hybridizing fragment is shown on the right side of the gel.

(B) Southern Blot analysis of the wild type (WT) and *mptpA* mutant (MT1 and MT2) strains of *M. tuberculosis*.

Genomic DNAs (3μg) from the wild type (WT) and *mptpA* mutant strain (MT1 and MT2) of *M. tuberculosis* were digested with *Pvu* II, separated on 1.2% agarose gel, transferred to Hybond N membrane and probed with ^{32}P labeled *mptpA* DNA fragment. The size of the DNA standards are shown on the left side of the gel and the size of hybridizing fragment is shown on the right side of the gel.

(C) Immunoblot analysis of the expression of MptpA in the wild type (WT) and *mptpA* mutant (MT1 and MT2) strains of *M. tuberculosis*.

Analysis of expression of MptpA in the wild type and *mptpA* mutant strain of *M. tuberculosis* by immunoblotting. The strains were grown in 7H9 media to mid-log phase. Equal amounts of whole cell lysate protein (40μg) was resolved on 12.5% SDS-PAGE, transferred to Hybond C Extra membrane and expression of MptpA was analysed by using polyclonal sera raised against MptpA in rabbits.

Figure 2: Survival of the wild type and *mptpA* mutant strains of *M. tuberculosis* in resting and activated macrophages.

The mouse macrophage cell line J774A.1 was infected with the wild type and *mptpA* mutant strain of *M. tuberculosis* separately at an MOI of 1:10 (macrophage: bacilli). At different time points post-infection (day 0, 2, 4, 6 and 8), macrophages were lysed and the number of intracellular mycobacteria was assessed by plating on 7H10 plates (A – in resting macrophages, B – in activated macrophages). The experiments were carried out twice in duplicates and data is depicted as mean of all four values ± S.E.

Figure 3: Bacterial load in spleens of animals infected subcutaneously with 5 x 10^7 cfu of either the wild type (WT) or *mptpA* mutant (MT) or complemented strain (CT) of *M. tuberculosis* and euthanised at 3 weeks (A) and 6 weeks (B) post – infection.

Spleens were homogenized in 5 ml of distilled water and ten- fold serial dilutions of the spleen homogenates were plated in duplicates on LJ slopes. Splenic bacillary load of animals euthanised at 3 weeks (A) and 6 weeks (B) post- infection was determined, converted to $\log_{10}$ cfu and depicted as mean ± S.E on y- axis. Various antigens are depicted on the x – axis.

Figure 4: Bacterial load in lungs of animals infected subcutaneously with 5 x 10^7 cfu of either the wild type (WT) or *mptpA* mutant (MT) or complemented strain (CT) of *M. tuberculosis* and euthanised at 3 weeks (A) and 6 weeks (B) post – infection.

A portion of lungs were homogenized in 5 ml of distilled water and ten- fold serial dilutions of the lung homogenates were plated in duplicates on LJ slopes. Lung bacillary load of animals euthanised at 3 weeks (A) and 6 weeks (B) post- infection was determined, converted to $\log_{10}$ cfu and depicted as mean ± S.E on y- axis. Various antigens are depicted on the x – axis.

Figure 5: Histopathology of liver and lung from guinea pigs infected subcutaneously with 5 x 10^7 cfu of either the wild type (WT) or *mptpA* mutant (MT) or complemented strain (CT) of *M. tuberculosis* and euthanised 3 weeks post- infection.

Portions of liver and lungs were removed under aseptical conditions and fixed in 10% formalin. Five-micron sections of tissues were stained with haematoxylin and eosin and subjected to histopathological analysis at a magnification of 10X. Representative sections of liver (A) and lung (B) from all the three groups of animals are shown. Sections of liver and lung from uninfected guinea pig were used as reference for normal tissue histology.

Figure 6: Histopathology of liver and lung from guinea pigs infected subcutaneously with 5 x 10^7 cfu of either the wild type (WT) or *mptpA* mutant (MT) or complemented strain (CT) of *M. tuberculosis* and euthanised at 6 weeks post - infection.

Sections (5μm) of liver and lung from animals infected with the wild type, *mptpA* mutant and complemented strains of *M. tuberculosis* were fixed, processed, stained with haematoxylin and eosin and observed under microscope at a magnification of 10X. Representative sections of liver (A) and lung (B) from all the three groups of animals are shown. Sections of liver and lung from uninfected guinea pig were used as reference for normal tissue histology.

Figure 7: (A) Southern blot analysis of the wild type and *mptpB* mutant strains (MT1, MT2 and MT3) of *M. tuberculosis*.

Genomic DNAs (3μg) from wild type (WT) and *mptpB* mutant strain (MT1, MT2 and MT3) of *M. tuberculosis* was digested with *Not* I, separated on 1.2% agarose gel, transferred to Hybond N membrane and probed with ^{32}P labeled *mptpB* DNA fragment. The size of DNA standards are shown on the left side of the gel and size of hybridizing band on the right side of the gel.

(B) Immunoblot analysis of the expression of MptpB in wild type (WT) and *mptpB* mutant (MT1, MT2 and MT3) strains of *M. tuberculosis*.

Analysis of the expression of MptpB in wild type and *mptpB* mutant strain of *M. tuberculosis* by immunoblotting. The strains were grown in 7H9 media to mid-log phase. Equal amounts of whole cell lysate protein (40μg) was resolved on 12.5% SDS-PAGE, transferred to Hybond C Extra membrane, the blot was probed for the expression of MptpB using polyclonal sera raised against MptpB in rabbits.

Figure 8: Survival of wild type and *mptpB* mutant strains of *M. tuberculosis* in macrophages.

The mouse macrophage cell line J774A.1 was infected separately with wild type and *mptpB* mutant strain of *M. tuberculosis* at an MOI of 1:10 (macrophage: bacilli). At different time points post-infection (day 0, 2, 4, 6 and 8), macrophages were lysed and the number of intracellular mycobacteria was assessed by plating on MB7H10 plates (A – in resting macrophages, B – in activated macrophages). The experiments were carried out twice in duplicates and data is depicted as mean of all four values ± S.E.

Figure 9: Total post mortem score of guinea pigs infected with 5 x 10^5 cfu of wild type (WT), *mptpB* mutant (MT) and complemented strain (CT) and euthanised at 3 weeks (A) and 6 weeks (B) post – infection.

At the time of sacrifice, depending on the magnitude of pathological damage in spleen, liver, lung, lymph nodes and sites of injection, scores were assigned to each organ as described by Mitchison. Total score for each animal was obtained by totaling up the scores obtained for individual organs and is depicted as mean ± S.E on y - axis. Various antigens are depicted on x - axis.

Figure 10: Bacterial load in spleens of guinea pigs infected with 5 x 10^5 cfu of either wild type (WT), *mptpB* mutant (MT) or complemented strain (CT) of *M. tuberculosis* and euthanised 3 weeks (A) and 6 weeks (B) post – infection.

Spleens were homogenized in 5 ml of distilled water and ten- fold serial dilutions of the spleen homogenates were plated in duplicates on LJ slopes. Splenic bacillary load of animals euthanised 3 weeks (A) and 6 weeks (B) post- infection was determined, converted to $\log_{10}$ cfu and depicted as mean ± S.E on y- axis. Various antigens are depicted on the x – axis.

Figure 11: Histopathology of liver and lung from guinea pigs infected with 5 x 10^5 cfu of either wild type, *mptpB* mutant or complemented strain of *M. tuberculosis* and euthanised at 3 weeks post- infection.

Portions of liver and lungs were removed under aseptical conditions and fixed in 10% formalin. Five-micron sections of tissues were stained with haematoxylin and eosin and subjected to histopathological analysis at a magnification of 10X. Representative sections of liver (A) and lung (B) from all the three groups of animals are shown.

Sections of liver and lung from uninfected guinea pig were used as reference for normal tissue histology.

Figure 12: Histopathology of liver and lung from guinea pigs infected with 5 x 10^5 cfu of either wild type, *mptpB* mutant or complemented strain of *M. tuberculosis* and euthanised at 6 weeks post - infection.

Sections (5µm) of liver and lung from animals infected with wild type, *mptpB* mutant and complemented strains of *M. tuberculosis* were fixed, processed, stained with haematoxylin and eosin and observed under microscope at a magnification of 10X. Representative sections, with an inset of high magnification (20X), of liver (A) and lung (B) from all the three groups of animals are shown. Sections of liver and lung from uninfected guinea pig were used as reference for normal tissue histology.

Examples:

The following methods are listed to illustrate the invention and should not be construed to limit the scope of the invention.

Example 1: Source of reagents and chemicals used:

Reagents, chemicals and enzymes including media for growing culture were purchased from standard sources.

Example 2: Culturing of bacterial strains (*E.coli* and Mycobacteria)

E. coli was grown in either Luria Bertani medium or in 2XYT medium supplemented with either of the antibiotics; ampicillin (50 µg/ml); kanamycin (25 µg/ml); gentamycin (50 µg/ml) or hygromycin (150 µg/ml). *M. tuberculosis* Erdman was grown in Middlebrook 7H9 medium supplemented with 0.5% glycerol, 0.2% Tween-80 and 1X ADC supplement. The cultures were grown with constant shaking at 200 rpm, 37°C. Solid media included LB Agar in case of *E. coli* and 7H10/7H11 media containing 0.5% glycerol, 1XOADC supplement and appropriate antibiotics in case of *M. tuberculosis*.

Example 3: Isolation of genomic DNA from mycobacteria:

Mycobacteria was grown to an A_{600nm} of 2-3 and glycine was added to the culture at a final concentration of 1%. 24 hours after addition of glycine, cells were harvested by centrifugation at 8,000 rpm for 10 minutes at room temperature. The pellet was resuspended in 500 µl of TEG solutions and 50 µl of lysozyme (20 µg/ml) was added.

After overnight incubation at 37°C, lysis was carried out by the addition of 100 µl of 10% SDS and 50 µl of Proteinase K (10 mg/ml) followed by incubation at 55°C for 40 minutes. To the cell lysate, 200 µl of NaCl and 160 µl of CTAB was added and the suspension was incubated at 65°C for 10 minutes. The lysate was extracted twice with phenol (pre-equilibrated with Tris-Hcl, pH 8.0) and twice with chloroform. The DNA was precipitated by adding 1/10th volume of 3M sodium acetate and two volumes of chilled ethanol. The DNA pellet was then washed with 70% ethanol and resuspended in 100 µl of autoclaved double distilled water.

Example 4: Polymerase Chain Reaction (PCR):

Amplification of genes by PCR was carried out as per manufacturer's recommendations. All PCR reactions were performed by using Taq/Pfu mix. The sequences of oligonucleotides used are shown in Table 3. A typical amplification reaction contained 10 ng of template DNA, 1x Taq polymerase buffer, 200 µM dNTPs, and 20 pmoles each of forward and reverse primers, 1.5 mM $MgCl_2$ and 1U of Taq/Pfu mix (Taq and Pfu DNA polymerase were mixed in a ration of 9:1). The amplification reactions to amplify various DNA fragments are shown in Table 4.

A typical amplification reaction comprised of;

1. Initial denaturation at 94°C for 5 minutes.
2. 30 cycles of denaturation at 94°C for 45 seconds, annealing at 55°C - 65°C for 1 minute and extension at 72°C for 1 minute. .
3. Final extension at 72°C for 10 minutes.

The PCR products were resolved on 1.2 % agarose gel and purified by using Qiagen gel extraction kit, as described above.

Transformation of *E. coli*:

E. coli XL-1 Blue and *E. coli* HB101 strains were grown in LB medium and competent cells were prepared by using the $CaCl_2$ method (Sambrook *et al.*, 1989). For preparation of high efficiency transformation cells, *E. coli* strains were grown to an A_{600nm} of 0.4 - 0.6 at 30°C and chilled at 4°C for 2 hours. The cells were harvested by pelleting the culture at 6,000 rpm at 4°C for 15 minutes. The cell pellet was resuspended in ice-cold trituration buffer (1/20th of the original culture volume) and diluted to the original culture volume by using prechilled tituration buffer. After incubating on ice for 45 minutes, cells were harvested by centrifugation at 6,000 rpm for 10 minutes at 4°C. The cell pellet was gently resuspended on ice-cold trituration buffer (1/10th of the original volume). Glycerol was added drop wise with gentle

swirling to a final concentration of 15% (v/v) and competent cells were stored in aliquots of 1ml each at –70°C, till further use.

Transformation was carried out by the method described by Mandel and Higa (Mandel and Higa, 1970). The ligations or supercoiled DNA were mixed with 200 µl of cells and incubated on ice for 30 minutes. Cells were then subjected to heat shock at 42°C for 45 seconds, followed by incubation on ice for 2 minutes. After incubating on ice, 800 µl of LB medium was added to the cells and the sample was incubated at 37°C for one hr with constant shaking at 200rpm. The transformants were selected on LB agar plates supplemented with the appropriate antibiotic(s).

Example 6: Preparation of plasmid DNA from *E. coli* transformants This was carried out as per following protocols separately:

A) Mini-preparation of Plasmid DNA

(i) By alkaline lysis - method:

A single colony was inoculated in 3 ml of 2XYT medium containing appropriate antibiotic(s) and grown overnight at 37°C with shaking at 200 rpm. The cells were harvested by centrifugation at 6,000 rpm for 2 minutes at 4°C. The cell pellet was resuspended in 200 µl of TEG solution containing lysozyme (to a final concentration of 20 µg/ml) and the suspension was incubated at room temperature for 10 minutes. After incubating for 10 minutes 400 µl of freshly prepared alkaline - SDS solution was added followed by mixing and gentle inversion. After incubating on ice for 5 minutes, 300 µl of 3M potassium acetate was added, mixed by inversion and further incubated on ice for 10 minutes. The cell lysate was subjected to centrifugation at 12,000 rpm for 15 minutes at 4°C, followed by phenol chloroform extraction, followed by chloroform extraction, precipitated by adding 540 µl of isopropanol (0.6v/v) and DNA followed by centrifugation at 12,000 rpm for 10 minutes at room temperature. The pellet was washed twice with chilled 70% ethanol, air-dried and resuspended in 50 µl of TE buffer.

(ii) By boiling lysis method:

The bacterial culture was grown and harvested as described above. The cell pellet was resuspended in 600 µl of STET solution containing lysozyme (to a final concentration of 20 µg/ml). After incubating for 15 minutes at room temperature, the cell suspension was boiled at 100°C for 2 minutes. The clarified cell lysate was prepared by subjecting the crude cell lysate to centrifugation at 12,000 rpm for 15 minutes at room temperature. The DNA was precipitated by adding 600 µl of

ammonia mix solution and recovered by centrifugation at 12,000 rpm for 10 minutes at room temperature. The pellet was washed twice with chilled 70% ethanol, air-dried and resuspended in 50 µl of TE buffer.

(iii) **By Qiagen miniprep kit:**

The bacterial culture was grown and harvested as described above. The pellet was resuspended in 250 µl of buffer P1 and incubated at room temperature for 5 minutes. After incubating for 5 minutes, 250 µl of buffer P2 was added and mixed by gentle inversions. After incubating for 5 minutes, 350 µl of buffer N3 was added and incubated on ice for 5 minutes and the clarified cell lysate was prepared by centrifugation at 12,000 rpm at 4°C for 15 minutes. The supernatant was passed through the Qia column, followed by washing with 500 µl of buffer PB. The column was then washed twice with 750 µl of buffer PE. The purified DNA was eluted in 100 µl of elution buffer.

B) Maxi preparation of DNA:

Plasmid DNA was isolated on a large scale by the alkaline SDS method (Sambrook *et al* 1989). A single colony was inoculated in 200 ml of 2XYT medium containing appropriate antibiotic(s) and grown overnight at 37°C with shaking at 200 rpm. The cells were harvested by centrifugation at 6,000 rpm for 15 minutes at 4°C. The cell pellet was resuspended in 4 ml of Solution I containing lysozyme (to a final concentration of 20 µg/ml). The sample was incubated on ice for 30 minutes. After incubating on ice for 30 minutes, 8 ml of freshly prepared Solution II was added and the sample was further incubated on ice for 15 minutes. Then, 6 ml of Solution III was added and incubated on ice for 10 minutes. The clarified cell lysate was prepared by centrifugation at 12,000 rpm for 15 minutes at 4°C. The DNA was precipitated from the cell lysate by addition of 10.8 ml of isopropanol (0.6v/v). After incubating at room temperature for 10 minutes, plasmid DNA was recovered by centrifugation at 12,000 rpm for 15 minutes at room temperature. The pellet was washed twice with chilled 70% ethanol, air-dried and resuspended in 750 µl of TE buffer. The DNA was incubated with RNAaseA (20 µg/ml) for 30 minutes at 37°C, followed by extraction with phenol chloroform. DNA in the aqueous phase was precipitated by addition of 2.5 volumes of chilled absolute ethanol and sodium acetate to a final concentration of 0.3M. The DNA was incubated at –70°C for 15 minutes, and DNA was recovered by centrifugation at 12,000 rpm for 15 minutes at 4°C. The pellet was washed twice with 70% ethanol, air-dried and resuspended in 100 µl of TE buffer.

Example 7: DNA manipulations for Cloning Purposes:

Restriction Digestion of DNA:

The restriction enzyme digestions of DNA were carried out at the specified temperature, as per manufacturer's recommendations. The analytical digestion was carried out in a reaction volume of 20 µl and preparative digestions were carried out in a reaction volume of 100 µl.

Dephosphorylation of DNA termini:

Removal of 5' phosphate groups from DNA fragments was carried out by using Calf intestinal phosphatase. The DNA was incubated with the enzyme (1U) in 1X buffer at 37°C for 30 minutes followed by incubation at 56°C for 30 minutes. The enzyme was inactivated by incubating the reaction mixture at 65°C for 10 minutes followed by phenol chloroform extraction and DNA was ethanol precipitated and resuspended in 10 µl of autoclaved double distilled water.

End filling of 5' overhang of DNA fragment:

DNA fragment with 5' overhang was end repaired by using Klenow fragment of DNA polymerase-I. The DNA (50ng/µl) was incubated with the enzyme (1-2U per µg of DNA) in 1X buffer containing 200 µM of dNTPs and incubated at 25°C for 15 minutes, followed by heat inactivation at 75°C for 15 minutes.

Ligation of DNA termini:

All the ligation reactions were carried out in a volume of 10 µl at 25°C for 3-4 hours. Each reaction contained typically 100 ng of the digested vector DNA, insert DNA fragment at 1:3 and 1:5 (vector: insert) molar concentrations and 1x ligase buffer containing 1mM ATP and 40U of T4 DNA ligase. The ligation mixtures were then used to transform competent cells of *E. coli* XL1-Blue and transformants were selected on appropriate LB agar supplemented with appropriate antibiotic(s).

Example 8: Agarose Gel Electrophoresis:

Agarose gel electrophoresis was carried out essentially as described earlier (Sambrook *et al.*, 1989). DNA fragments of size > 500 bp were resolved on 0.8% agarose gel, while those in the range of 250 - 500 bp were resolved on 1.2% agarose gel. The gels were electrophoresed in 1X TAE buffer containing 0.5 µg/ml ethidium bromide.

Example 9: Elution of DNA from Agarose:

DNA was eluted from agarose gel by using the Qiagen gel extraction kit. The gel was excised out and incubated with 3 gel volumes of QG buffer, at 55°C till the agarose was melted. The samples were then passed through Qia column, column was washed twice with PE buffer and the DNA was eluted in 50 µl of elution buffer.

Example 10 : Immunoblot analysis:

Protein samples were resolved on 10% SDS - PAGE and then transferred to Hybond C extra membrane overnight at 40mA or at 180mA for 2hours by using the Bio-Rad mini Trans Blot Cell (Bio-Rad Laboratories,. Hercules, CA, USA). Transfer of the protein to the membrane was confirmed by staining with Ponceau S stain. The membrane was blocked in 2% milk for 2 hours at room temperature. The blot was than incubated with 1:10,000 dilution of the polyclonal sera for 2 hours at room temperature. To prevent non-specific binding of antibody, the dilutions were prepared in 2% milk-PBST. The blot was then washed thrice with PBST. After washing, the blot was incubated with peroxidase conjugated goat anti-rabbit Immunoglobulin-G at a dilution of 1:2500. After incubation for 1 hour, the blot was washed thrice with PBST and the immunoreactive bands were visualized by the addition of PBS containing 10 µl/ml of 30% H_2O_2 and 0.5 mg/ml 3,3' diaminobenzidine tetrahydrochloride.

Example11: DNA sequencing

The DNA samples for sequencing were prepared from 3ml culture of the respective transformants using the Qiagen prep spin plasmid kit. The DNA samples were sequenced by using an ABI Prism 377 sequencer with rhodamine dye terminator chemistry.

The sequencing PCR reaction was set up in a PE-2400 thermocycler (Perkin Elmer – Cetus, Norwalk, Connecticut, USA) by using 500 ng double stranded DNA and 3.2 pmol vector specific oligonucleotides. After completion of the sequencing reactions, the extension products were precipitated with sodium acetate and ethanol to remove un-incorporated terminators. The samples were than loaded onto a 4% long ranger gel. The sample lanes were analysed on a DNA sequencing analysis 3.0 software (ABI-Prism, Perkin Elmer Applied Biosystems, Foster City, CA, USA).

Example 12: Electroporation of *M. tuberculosis*:

M. tuberculosis cultures were grown to A_{600nm} of 0.8 with shaking at 200 rpm at 37°C. Before harvesting, the cells were chilled on ice for one hour. Cells were pelleted by centrifugation at 6,000 rpm at 4°C for 10 minutes, washed twice with chilled glycerol (10%), resuspended in 1 ml of chilled glycerol (10%) and stored in aliquots of 100 µl each at –70°C, till further use.

For electroporation, approximately 2 µg of DNA was mixed with 20 µl of cells, kept on ice for 15 minutes and cells were subsequently pulsed at field strength of 16 kV/cm (400 V input, 330µF capacitance, 8kOhms resistance, 2.4 kV output using cuvette with 0.15 cm gap width). Cells were recovered in 1ml of 7H9 medium at 37°C, 200 rpm for 24 hours. The transformants were selected on Middlebrook 7H10 agar plates supplemented with ADC and containing appropriate antibiotic(s). Plates were incubated for 14-21 days at 37°C.

Example13: Alkali and U.V. pretreatment of DNA:

The targeting DNA was pretreated with alkali before its electroporation into the competent cells of *M. tuberculosis* as per the method described by Hinds *et al* 1999 (Hinds *et al.*, 1999). The vector was denatured in 20 µl of 0.2M NaOH containing 0.2 mM EDTA for 30 minutes at 37°C. The denatured DNA was precipitated by addition of $1/10^{th}$ volume of 3M sodium acetate and 2.5 volumes of chilled absolute ethanol. The DNA was precipitated by incubating the samples at –70°C for 15 minutes and recovered by centrifugation at 12,000 rpm for 15minutes at 4°C. The pellet was washed twice with chilled 70% ethanol to remove salts, air dried and resuspended in 10 µl of double distilled water. For U.V. pretreatment, DNA was subjected to U.V. irradiation in an U.V. stratalinker 1800 (Amersham) at 100-mJ cm^{-2} for 5 minutes. For alkali and U.V. pretreatment of DNA, the DNA was prepared by Qiagen column as described above.

Example 14: Southern Blot hybridization:

The genomic DNA was isolated from *M. tuberculosis*, and subjected to restriction digestion by appropriate restriction endonuclease. The digested fragments were resolved on a 1.2% agarose gel at low voltage (40V) overnight in 1X TAE gel running buffer. The DNA fragments were depurinated by soaking the gel in 0.1N HCl for 10 minutes followed by a wash with double distilled water. The DNA was then denatured by soaking the gel in denaturation buffer (1.5M NaCl, 0.5 M NaOH). The gel was then rinsed with double distilled water and neutralized in neutralization buffer (1M Tris pH

7.4, 1.5 M NaCl). The DNA was then transferred to Hybond N membrane by capillary transfer in 20X SSC overnight (Southern 1975). The membrane was air-dried and DNA was cross-linked to the Hybond N membrane by U.V. irradiation for 2 minutes at 700mJ. The blot was prehybridized in a solution containing 50% deionised formamide, 5X SSC, 5X Denhardts solution, 50mM Tris-Cl, pH7.5 and 200 µg/ml denatured salmon sperm DNA overnight at 42°C. The heat denatured probe was then added to the blots and hybridization was carried out at 42°C for 14-16 hours. The blot was washed first in 2X SSC and 0.1% SDS at room temperature for 30 minutes and then in 0.2X SSC and 0.1% SDS at room temperature for 30 minutes and then in 0.2X SSC and 0.2% SDS at 65°C for 30 minutes. The blot was then air dried, wrapped in saran wrap and subjected to autoradiography.

Example 15: Preparation of Nucleic Acid Probes:

The DNA fragment to be labeled was PCR amplified by using gene specific primers. The amplicon was purified by using Qiagen gel extraction kit and end-labeled by using NEBlot kit in a 50 µl reaction. The labeling reaction comprised of 100ng of template DNA, 1x klenow buffer (having random primers), 1mM dGTP, 1mM dCTP, 1mM dTTP and 10uCi of α^{32}P dATP, 1U of klenow fragment. The template DNA was denatured at 100°C for 5 minutes and kept in ice for 2 minutes, dNTPs and enzyme were added and end labeling was carried out at 37°C for 2hours. Unincorporated dNTPs were removed by using Qiagen nucleotide removal kit and the labeled probe was added to the blot.

Example 16 : Preparation of Nucleic Acid Probes:

The DNA fragment to be labeled was PCR amplified by using gene specific primers. The amplicon was purified as described above and end-labeled by using NEBlot kit in a 50 µl reaction. The labeling reaction comprised of 100 ng of template DNA, 1x klenow buffer (having random primers), 1mM dGTP, 1mM dCTP, 1mM dTTP and 10 µCi of α^{32}P dATP (3500 Ci/ mmol), 1U of Klenow DNA polymerase I fragment. The template DNA was denatured at 100°C for 5 minutes and kept on ice for 2 minutes, dNTPs and enzyme were added and end labeling was carried out at 37°C for 2hours. Unincorporated dNTPs were removed by using Qiagen nucleotide removal kit and the labeled probe was added to the blot.

Example 17: *In vitro* infection of mouse macrophage cell line by *M. tuberculosis*:

J774A.1 mouse macrophage cell line (resting or activated with rIFN-γ 50Uml^{-1} for 16hours) was seeded in a six well plate at a density of 2x10^5 per well. Before infection, the cell lines were washed once with 1 x Hanks Balanced Salt Solution (HBSS) and medium was replaced with Dulbeccos modified eagle's medium (DMEM) supplemented with 10% heat inactivated fetal calf serum (FCS). The bacterial strains were washed twice with DMEM and resuspended in DMEM supplemented with 5% FCS. The cells were infected with wild type or mutant strain at an MOI of 1:10 (macrophage : bacteria). The cells were incubated at 37°C in a 5% CO_2 atmosphere. After 6 hours of infection, cells were washed twice with 1x HBSS and overlayed with 2 ml DMEM supplemented with FCS (10%), Antibiotic-antimycotic (1%) and amikacin (20 µg/ml). On days 0, 2, 4, 6 and 8, infected cells were lysed in 1 ml of 0.1% Triton X-100 for 15 minutes. The number of bacilli at different time points was determined by plating 10-fold serial dilutions in duplicates on MB 7H10 medium and incubating the plates at 37°C for 3 weeks.

Example 18: Virulence studies in guinea pigs:

The effect of disruption of tyrosine phosphatases on the virulence of *M. tuberculosis* was evaluated in the guinea pig model of experimental tuberculosis. This work was carried at Tuberculosis Research centre, Chennai. Random-Bred guinea pigs of the Duncan-Hartley strain in the weight range of 200 - 400g were obtained from National Center for Laboratory Animal Science (NCLAS), Hyderabad.

The guinea pigs were divided into groups of sixteen each. Each group comprised of 16 animals, 8 males and 8 females. The different groups of guinea pigs were challenged with one of the organisms mentioned below subcutaneously and 8 animals (4 males and 4 females) were euthanised at 3 weeks and 6 weeks post – challenge.

a) *M. tuberculosis* Erdman
b) *mptpA* mutant strain
c) *mptpA* complemented strain

] High Dose (5 x 10^7)

d) *M. tuberculosis* Erdman
e) *mptpB* mutant strain
f) *mptpB* complemented strain

] Low Dose (5 x 10^5)

All the organisms were coded and animals were subcutaneously challenged with all the coded preparations separately by using a 1ml tuberculin syringe with a 26 G needle.

After euthanasia the following investigations were carried out

1) Gross body weight of the animal.
2) Weight of infected organs – liver, spleen and lung.
3) Scores of the gross pathological damage to the organs (Post-mortem scores).
4) Viable count of the tubercle bacilli from spleen and lung (Bacterial enumeration).
5) Histopathological evaluation of liver and lung.

The gross body weight of the animals was measured at the time of beginning of the experiment, and at weekly intervals till euthanasia. Liver, lungs, spleens and lymph nodes were removed aseptically and the weight of the infected organs was measured. The bacterial load was enumerated in spleens and lungs. Portions of liver and lung tissues were fixed in 10% formalin for histopathological analysis of granuloma formation and cellular composition of granuloma.

***Post-mortem* scores:**

The virulence was measured based on the rate of progression of the disease in guinea pig as described by Mitichison. (Mitichison, 1964). At the post-mortem examination of the animals, the total extent of tuberculosis disease was assessed as a score ranging from.0 to 100. The extent of visible lesions in the organs were scored as described in Table 5. Average score for each group was calculated.

***Viable count* of the tubercle bacilli from the spleen and lung:**

The spleen and portion of lung was removed into a sterile, weighed grinding tube. Organs were homogenised in 5ml of double distilled water by using a teflon homogenizer. Ten fold serial dilutions (10^{-1}, 10^{-2},10^{-3},10^{-4}) were prepared in distilled water and 10µl of neat homogenate and various dilutions were inoculated in LJ slopes in duplicates. The LJ slopes were incubated at 37°C and readings for cfu were taken after 4 weeks and 6 weeks. The number of cfu per organ and an average organ cfu for each group was calculated. The sensitivity of this detection method was 500 bacilli.

Histopathology of liver and lungs:

The liver and lungs of the animals were removed and stored in preweighed jars containing 10% formaldehyde. Two bits of tissue (2cm x 2cm thickness) each from liver and lung were fixed in 10% formalin until further treatment. The organ bits were

washed in 70% alcohol and 95% alcohol for 2 hours each followed by treatment with isopropanol for 2 hours. In order to ensure complete dehydration of the tissue, the isopropanol treatment was repeated twice. The bits were then incubated in xylene for 15-20 minutes and finally embedded in molten paraffin wax. The paraffin embedded tissue portions was divided into 5 μm fine sections by using a microtome (Reichert, Germany) and fixed onto glass slides. Deparaffinization of the cut sections was carried out prior to staining. The slides were first immersed twice in xylene for 5 minutes each followed by treatment with isopropanol twice for 3 minutes each. The slides were finally treated with 95% alcohol for complete removal of traces of wax.

The sections were stained with hematoxylin and eosin for the presence of granuloma. The sections were washed in water and stained with hematoxylin for 5 minutes. Excess stain from the slide was removed by washing with distilled water. The slides were then counterstained with eosin solution for 1 minutes, washed with water and air-dried. For viewing the slides under the microscope, the slides were mounted using DPS mount and covered with a coverslip. The proportion of the granuloma and extent and type of cellular infiltration in the sections were microscopically assessed as described earlier (Ridley, 1977 and Jayashankar and Ramanathan, 1999). The tissue sections were analysed for following parameters to determine the effect of disruption of tyrosine phosphatases on the virulence of *M. tuberculosis*; size of typical granuloma; amount of caseous necrosis; relative number of neutrophils; macrophages; giant cells; epitheloid cells and lymphocytes; degree to which lymphocytes were organized in the granuloma and extent to which granuloma were organized. At least four different sections for each tissue were analyzed.

Statistical analysis:

Data are depicted as arithmetic mean ± standard error mean. Data were analyzed for statistical significance using the Student's t test. Differences between the various groups of guinea pig were considered significant if p values were <0.05.

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Claims:

1. A method for demonstrating the role of MptpA in the virulence of *M. tuberculosis in vivo*. The said method comprises:

   a) extracting the genomic DNA from *M. tuberculosis* and PCR amplification of *mptpA* along with its left and right flanking regions using gene specific primers of sequence IDs 1 to 4.

   b) sequencing the PCR amplicon and its cloning in a non-replicative suicidal vector.

   c) Disruption of *mptpA* in the genome of *M. tuberculosis* after electroporation of the non-replicative vector, pAKΔA into *M. tuberculosis* resulting in the development of mptpA mutant strain of *M. tuberculosis*.

   d) Infection of activated mouse macrophages J774A.1 by *M. tuberculosis* and its *mptpA* mutant separately and demonstrating that *mptpA* mutant is impaired in it's ability to survive in the activated macrophages.

   e) PCR amplification of *mptpA* using gene specific primers of sequence IDs 5 to 6. Sequencing of the PCR product and its cloning in *E. coli* – mycobacteria shuttle vector. Electroporation of the shuttle vector into the *mptpA* mutant strain resulting in the complemented strain of *M. tuberculosis*.

   f) injecting the guinea pigs of sound health with the wild type, *mptpA* mutant (from step c) and complemented strains of *M. tuberculosis* from step e,

   g) comparative analysis of survival of the wild type, *mptpA* mutant and complemented strain in guinea pigs to evaluate the role of MptpA in the pathogenesis of *M. tuberculosis*.

   h) Demonstration of a 90-fold reduction in the ability of *mptpA* mutant as compared to the parental strain to survive in guinea pigs as a proof for the

essential role of tyrosine phosphatase A (MptpA) in the pathogenesis of *M. tuberculosis*.

2. Specific novel nucleic acid primers of claim 1:

Sequence ID1: CCA TCA TGA CTG TGG AAC CTA TTC CTG TCG GCC

Sequence ID2: GGG CAT ATG GGC TCC ATT CGC CGG ACT CGC CG

Sequence ID3: GGG CAT ATG GGC TGG ATT CGC CGG ACT CGC CG

Sequence ID4: CCA TCA TGA GTC GGT TAC CCC CGT ATA GCC CGG

Sequence ID5: G CAT ATG CAT CTG TGA TCC GCT GCA CGT CAC ATT C

Sequence ID6: CCG AGG CGT TCA ACT CGG TCC GTT CCG CGC GCG AC

3. A method for demonstrating the role of MptpB in the virulence of *M. tuberculosis in vivo*. The said method comprises:

a) Extracting the genomic DNA from *M. tuberculosis* and PCR amplification of *mptpB* along with its left and right flanking regions using gene specific primers of sequence IDs 7 to 10.

b) Sequencing the PCR amplicon and its cloning in a non-replicative suicidal vector.

c) Disruption of *mptpB* in the genome of *M. tuberculosis* after electroporation of the non-replicative vector, pBKΔB into *M. tuberculosis* resulting in the development of *mptpB* mutant strain of *M. tuberculosis*.

d) Infection of activated mouse macrophages J774A.1 by *M. tuberculosis* and its *mptpB* mutant separately and demonstrating that *mptpB* mutant is impaired in it's ability to survive in the activated macrophages.

e) PCR amplification of *mptpB* using gene specific primers of sequence IDs 11 to 12. Sequencing of the PCR product and its cloning in *E. coli* – mycobacteria shuttle vector. Electroporation of the shuttle vector in *mptpB* mutant strain resulting in complemented strain of *M. tuberculosis*.

f) Injecting the animal of sound health with wild type, *mptpB* mutant (from step c) and complemented strains of *M. tuberculosis from* step e.

g) Comparative analysis of the survival of wild type, *mptpB* mutant and complemented strain in guinea pigs to evaluate the role of MptpB in the pathogenesis of *M. tuberculosis*.

h) Demonstration of a 70-fold reduction in the ability of *mptpB* mutant as compared to the parental strain to survive in guinea pigs as a proof for the essential role of tyrosine phosphatase B (MptpB) in the pathogenesis of *M. tuberculosis*.

4. Specific novel nucleic acid primers of claim 3:

Sequence ID7: CCA TCA TGA CGT CGT CTGACA AC GAG CGT CC

Sequence ID8: GGG CAT ATG GCA ACA CCC CGG CCG CCC GCT CG

Sequence ID9: GGG CAT ATG ACG CTC GGC TGT TTG CGG CAG CTC G

Sequence ID10: CCA TCA TGA CGG TGG GTC GCC CCG CGG TGC GG

Sequence ID11: CG CAT ATG CGA TGG CTG TCC GTG AAC TGC CGG G

Sequence ID12: CAC GCG TTC CTG CGA GCA GCA CCC CGC GCA TCC G

5. A method as claimed in claims 1 and 3 wherein the animal used in the *in vivo* virulence studies are selected from guinea pigs, mice and rabbit.

6. A method as claimed in claims 1 and 3 wherein, the bacterial load was estimated by counting colony-forming units (cfu) in the spleen of animals infected with the wild type and the mutant strains of *M. tuberculosis*.

7. A method as claimed in claims 1 and 3 wherein, the tissue damage was evaluated by granuloma formation in liver and lungs of animals infected with wild type and the mutant strains of *M. tuberculosis*.

Dated, this 9th day of July, 2003.

Rajeshwari

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Abstract

Present invention relates to two Protein Tyrosine phosphatases (MptpA and MptpB) of *Mycobacterium tuberculosis* as potential targets for developing anti-tubercular drugs. Utility of the invention is for its application in the identification and development of target specific new anti-tubercular drugs. The invention identifies both protein tyrosine phosphatases of *M. tuberculosis* as the virulence factors for *M. tuberculosis*. The present invention specifically relates to the cloning of genes for both tyrosine phophatases designated as MptpA and MptpB into a non – replicative vector, disruption of both tyrosine phosphatases in the genome of *M. tuberculosis* and demonstration of impaired survival of the mutant strains of *M. tuberculosis* in the activated macrophages as well as in guinea pigs. It further provides for nucleic acid sequences of *mptpA* and *mptpB* along with their respective flanking regions, non- replicative vectors containing such nucleic acid sequences, *mptpA* mutant strain of *M. tuberculosis*, *mptpB* mutant strain of *M. tuberculosis*, *mptpA* complemented strain and *mptpB* complemented strains of *M. tuberculosis*.

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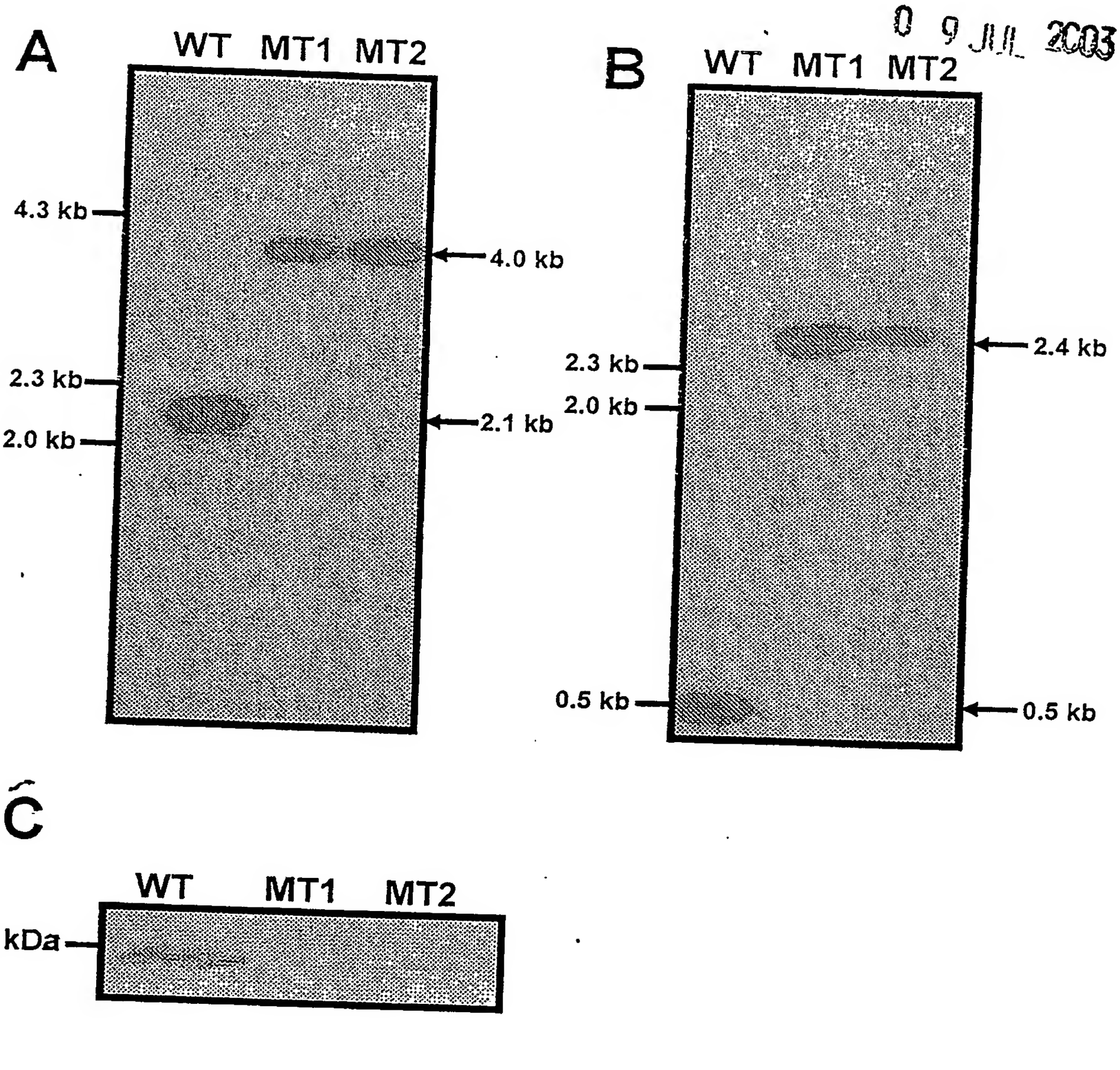

Fig. 1

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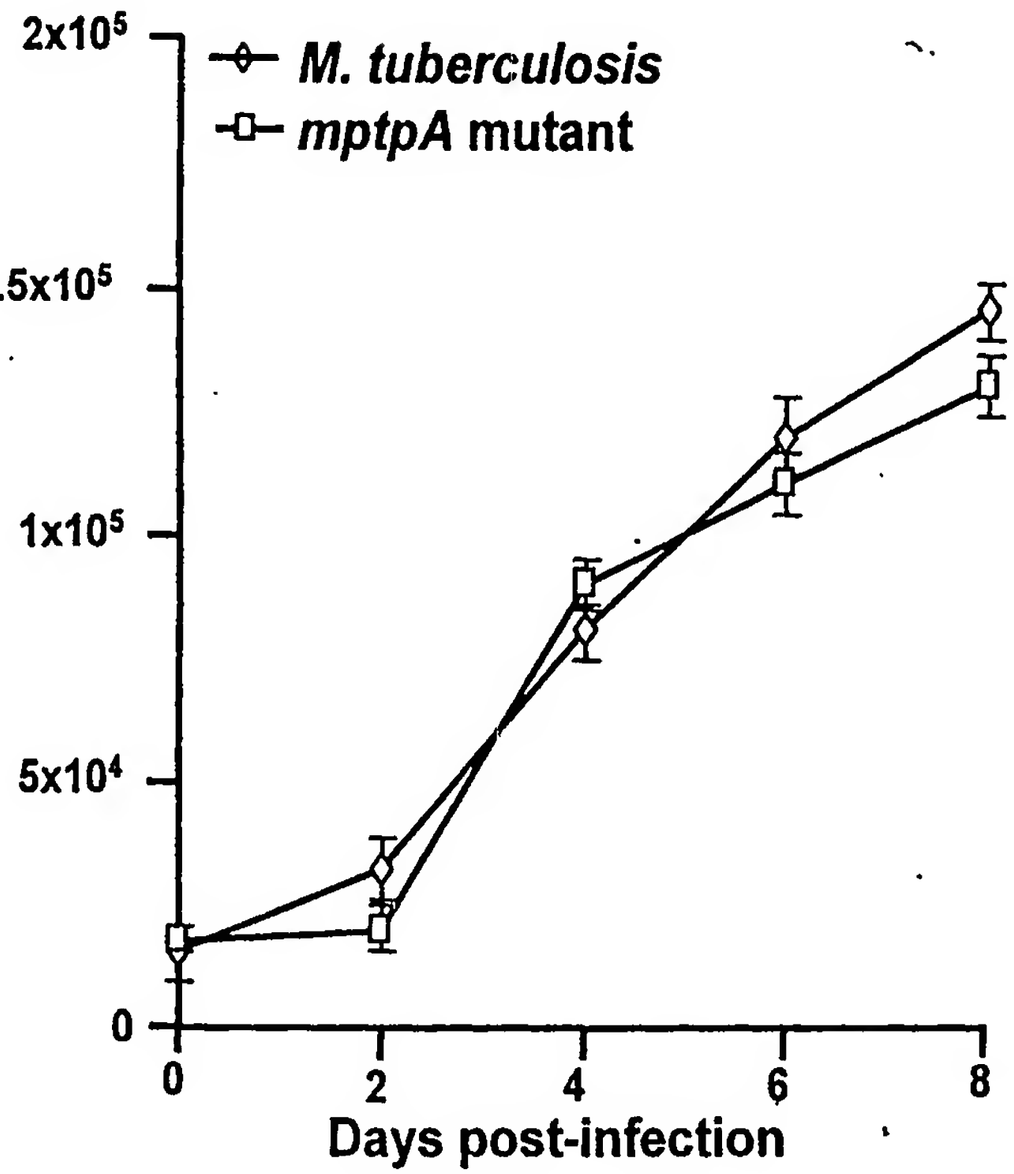

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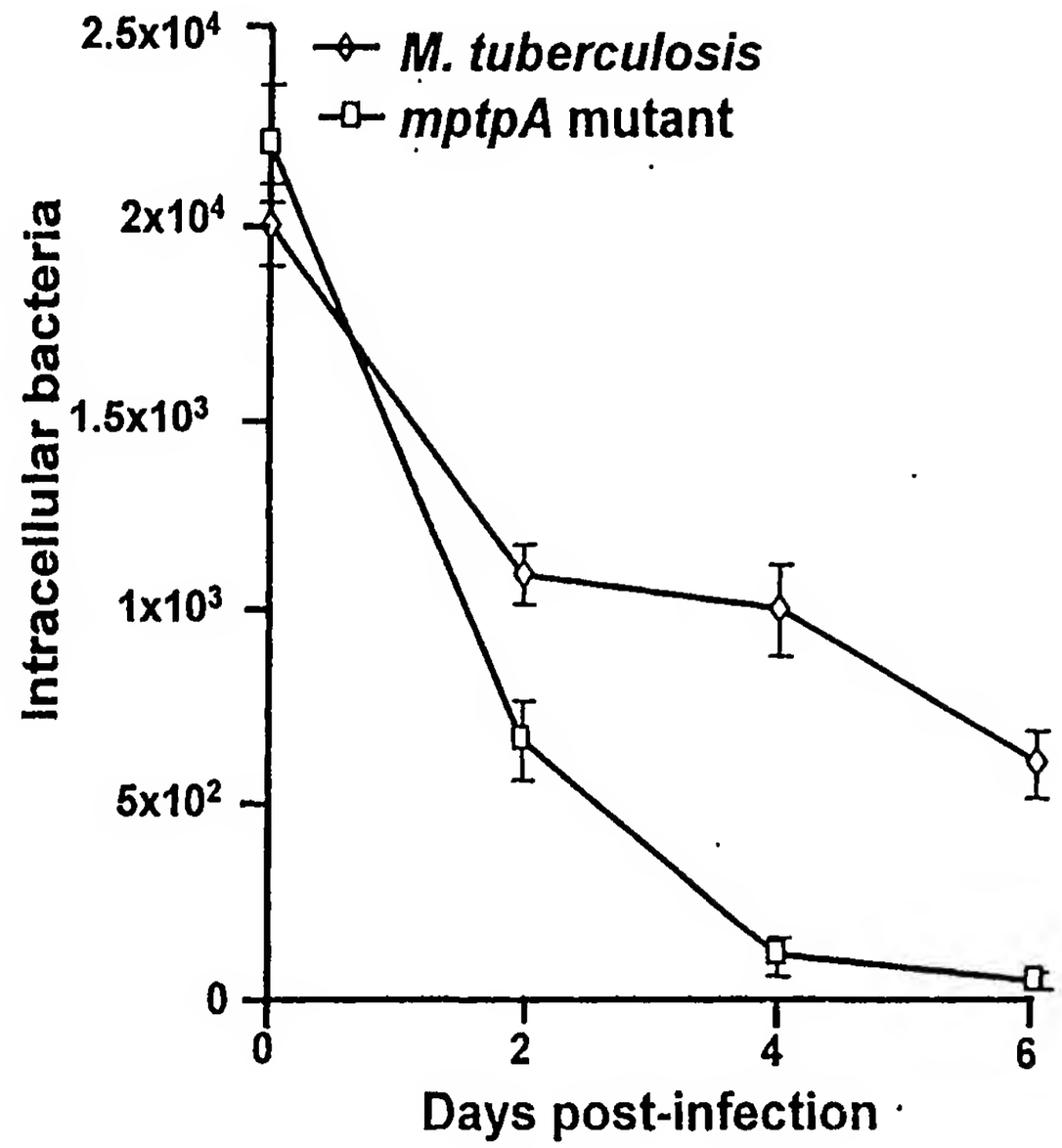

Fig. 2

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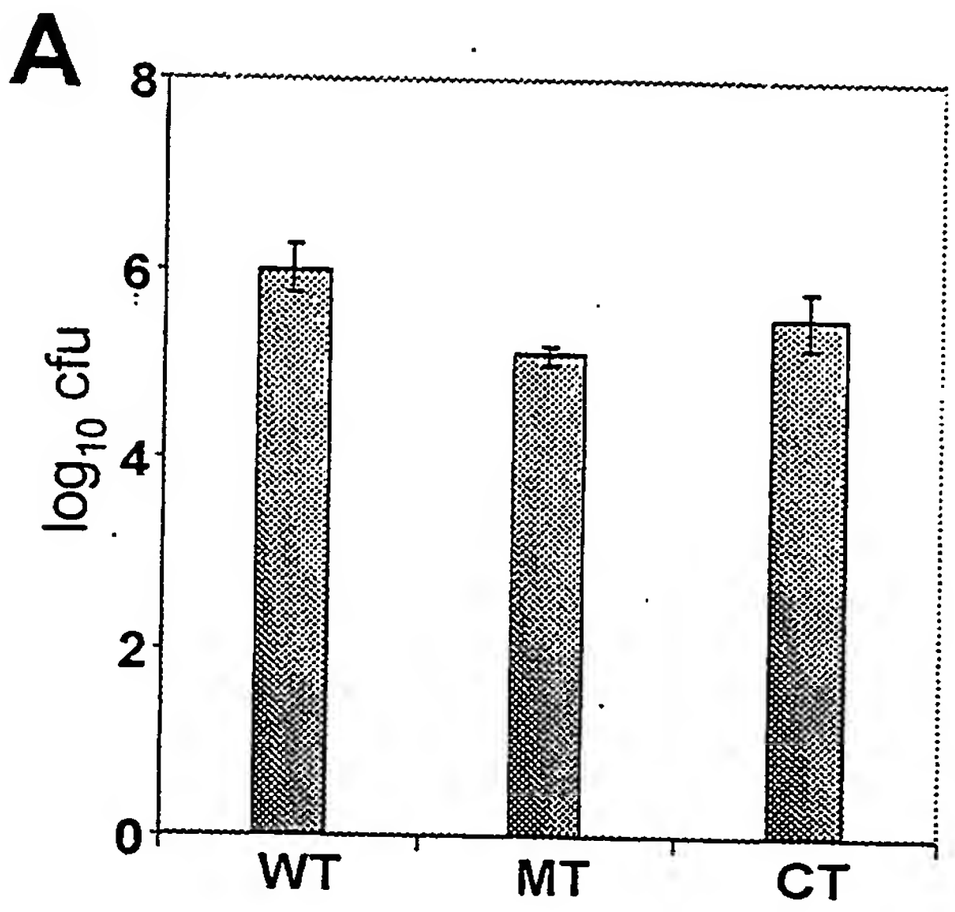

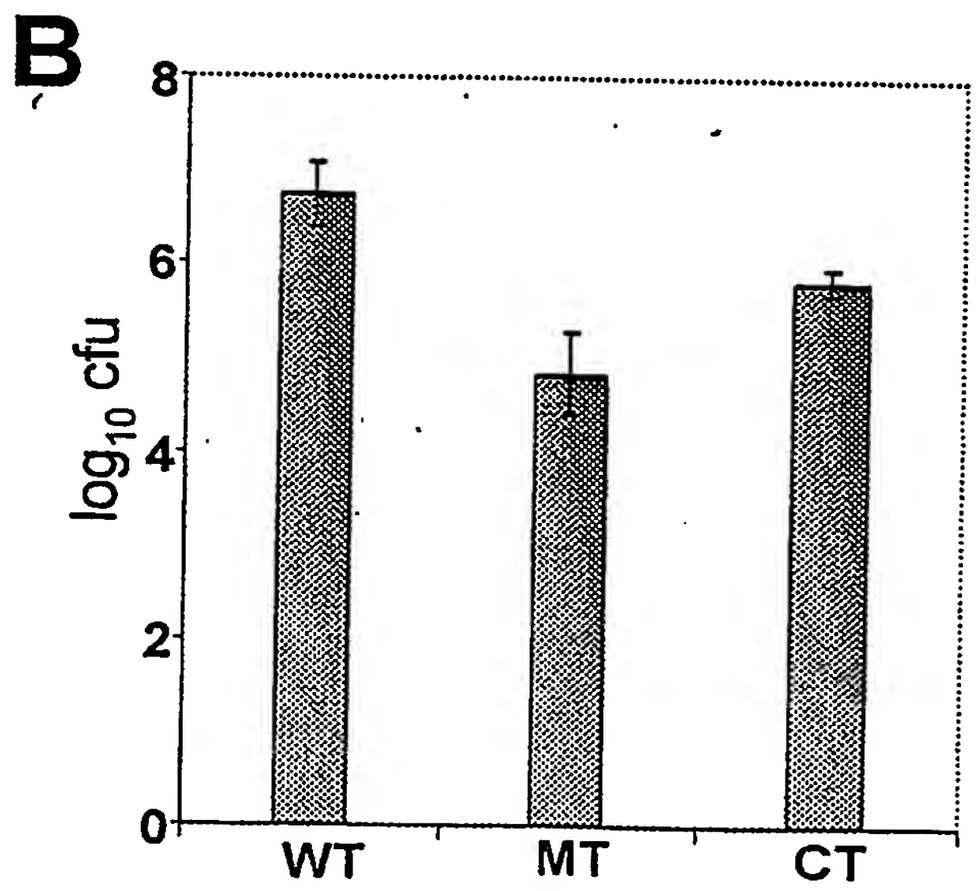

Fig. 3

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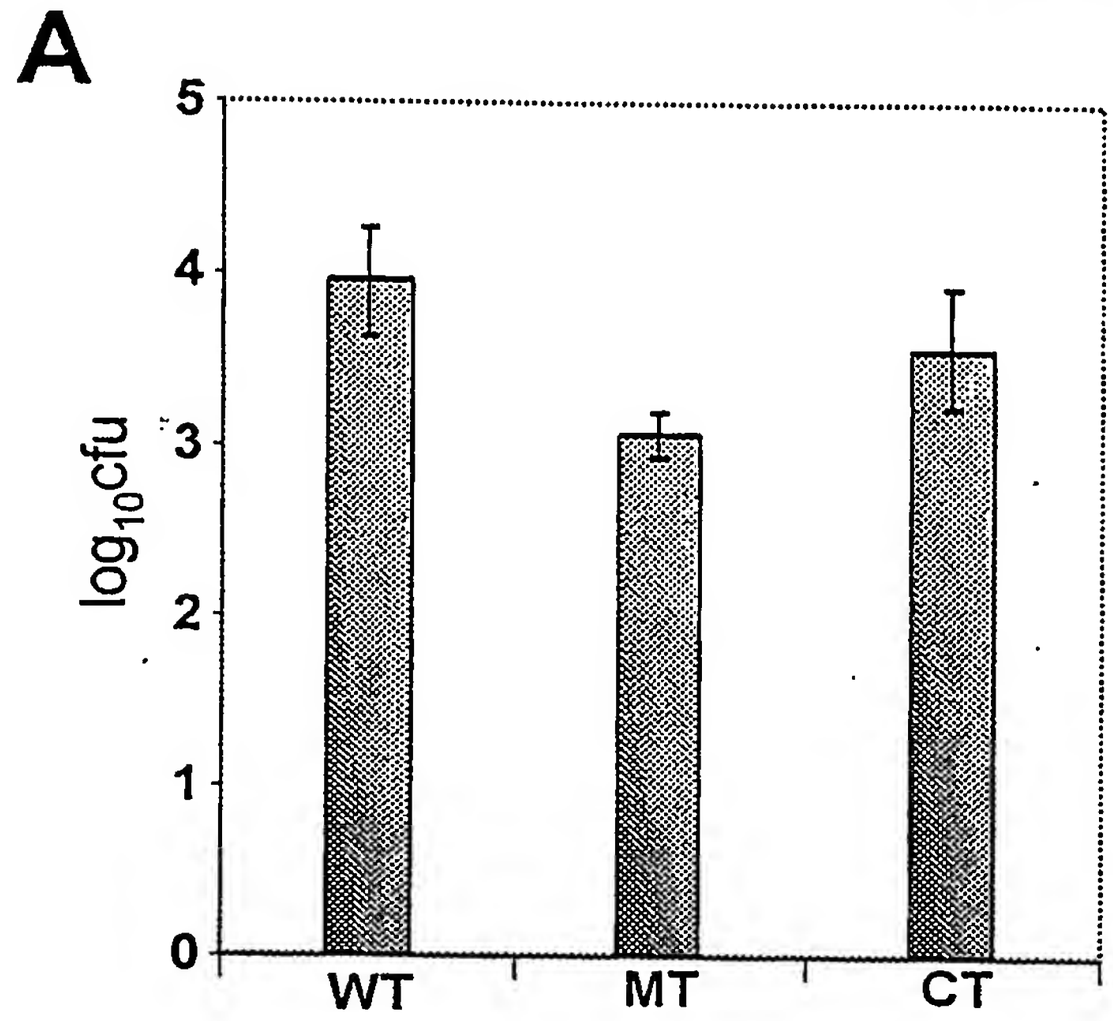

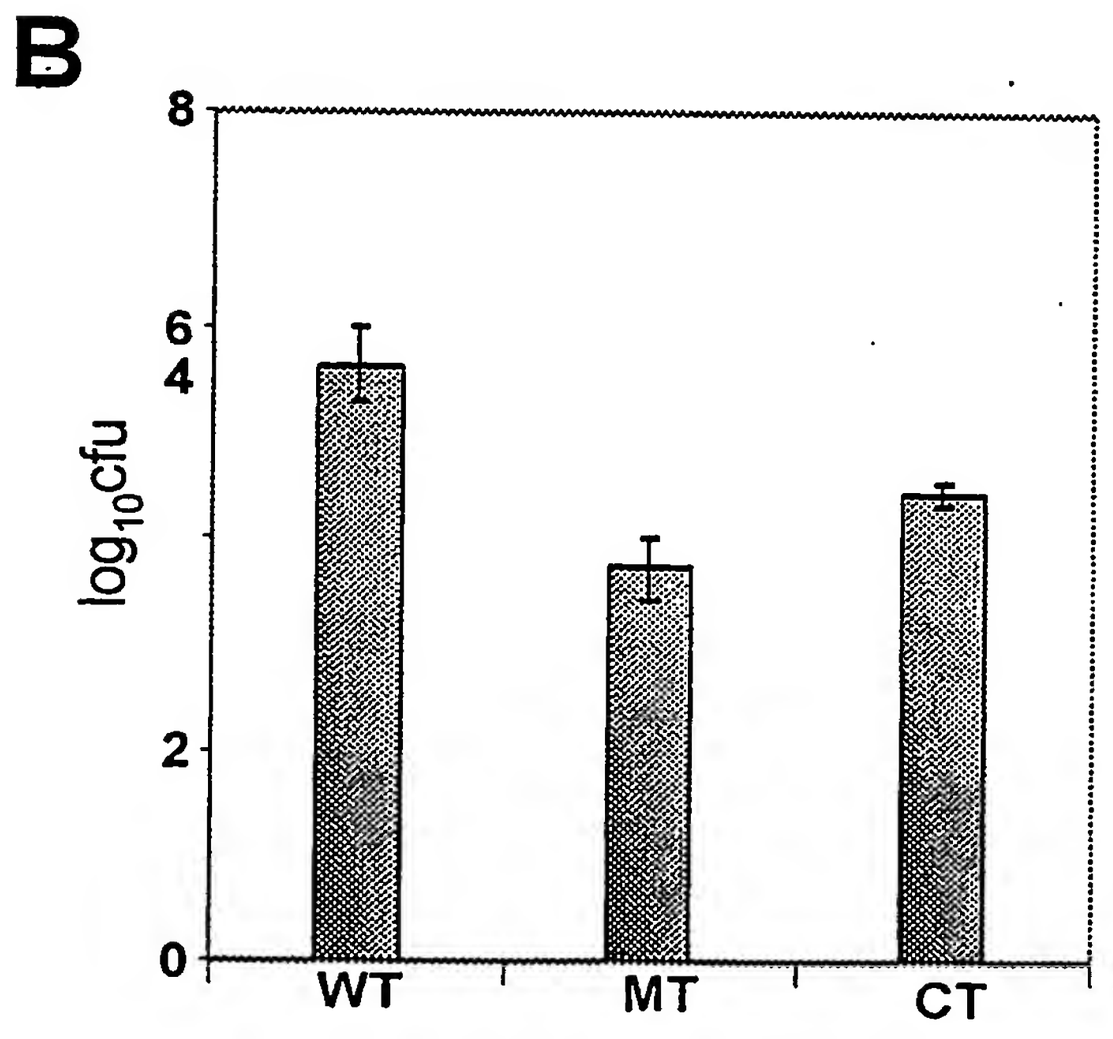

Fig.4

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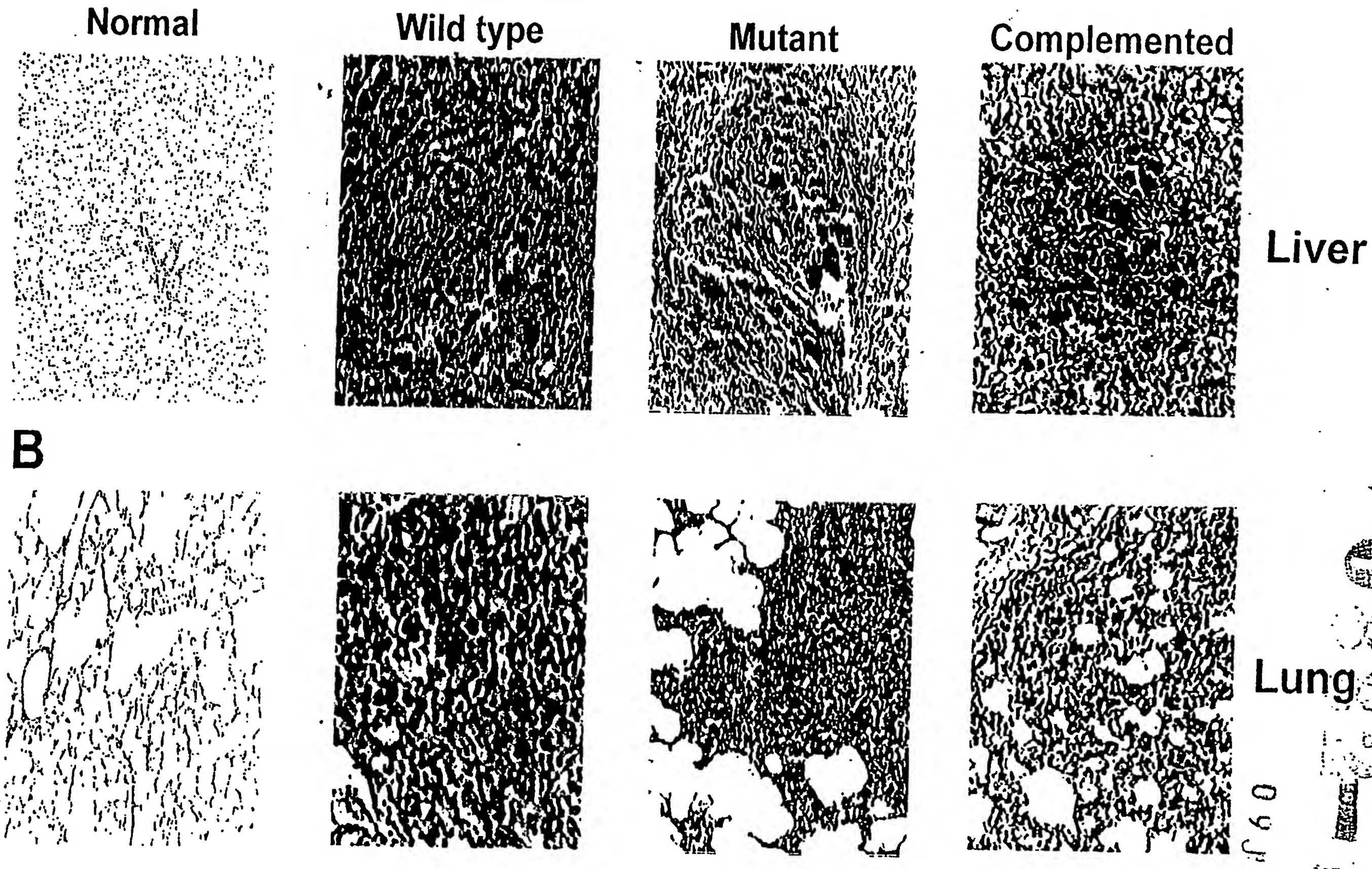

Fig. 5

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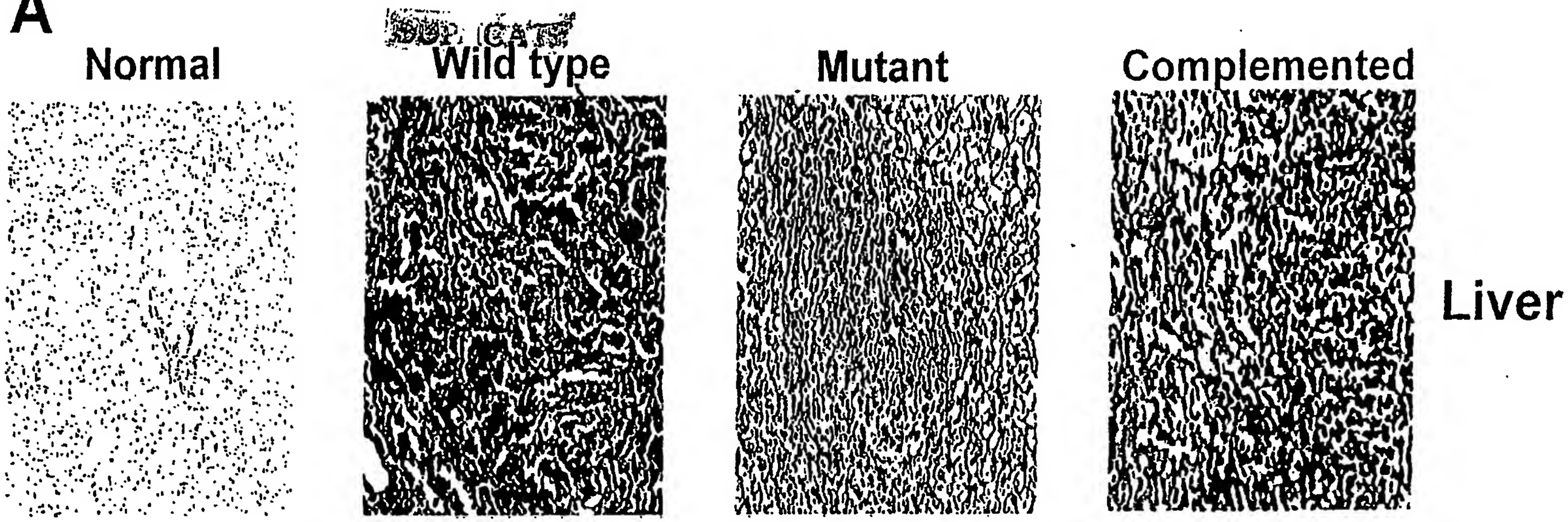
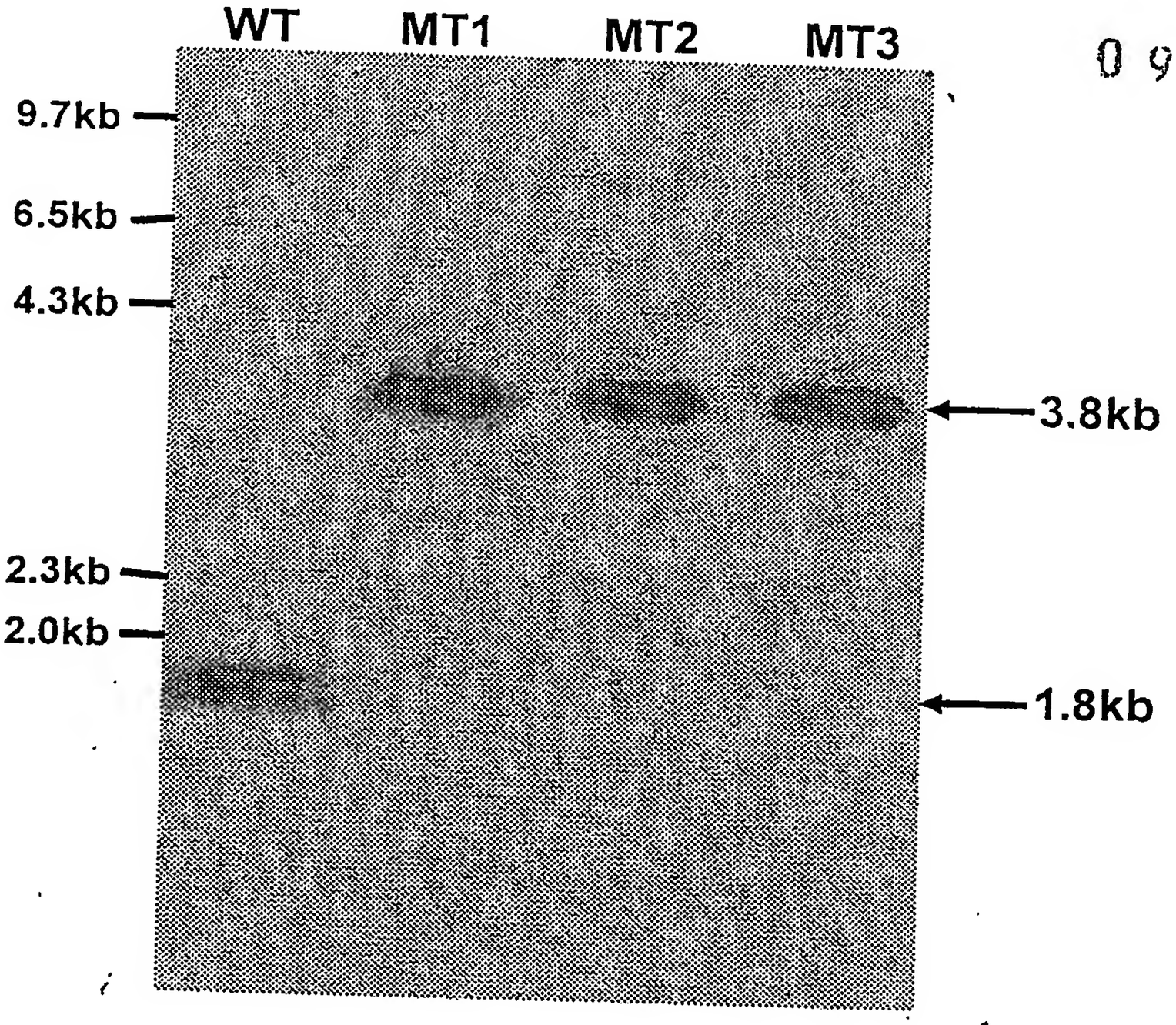


Fig. 6



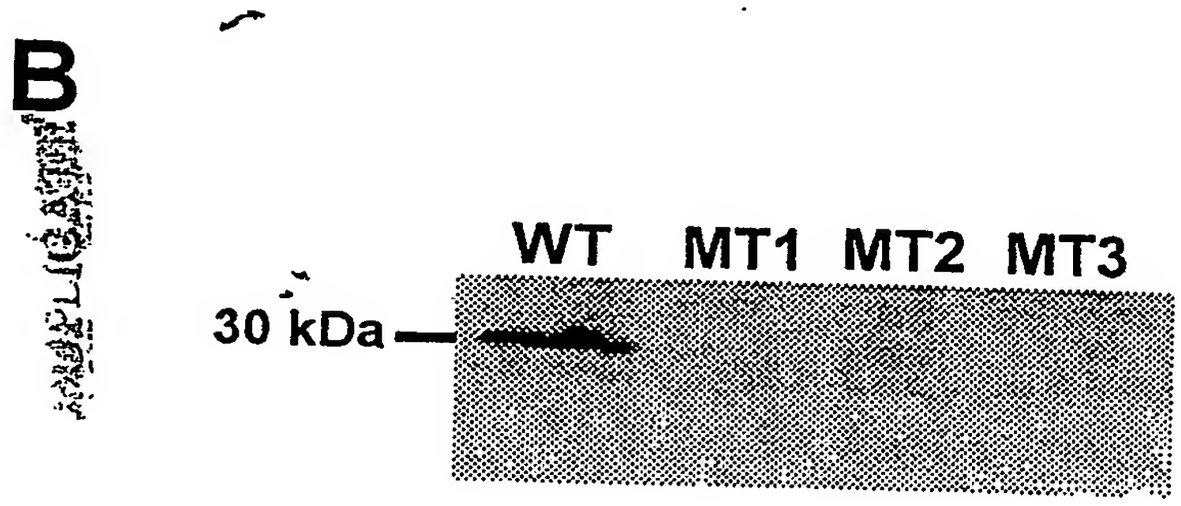


Fig. 7

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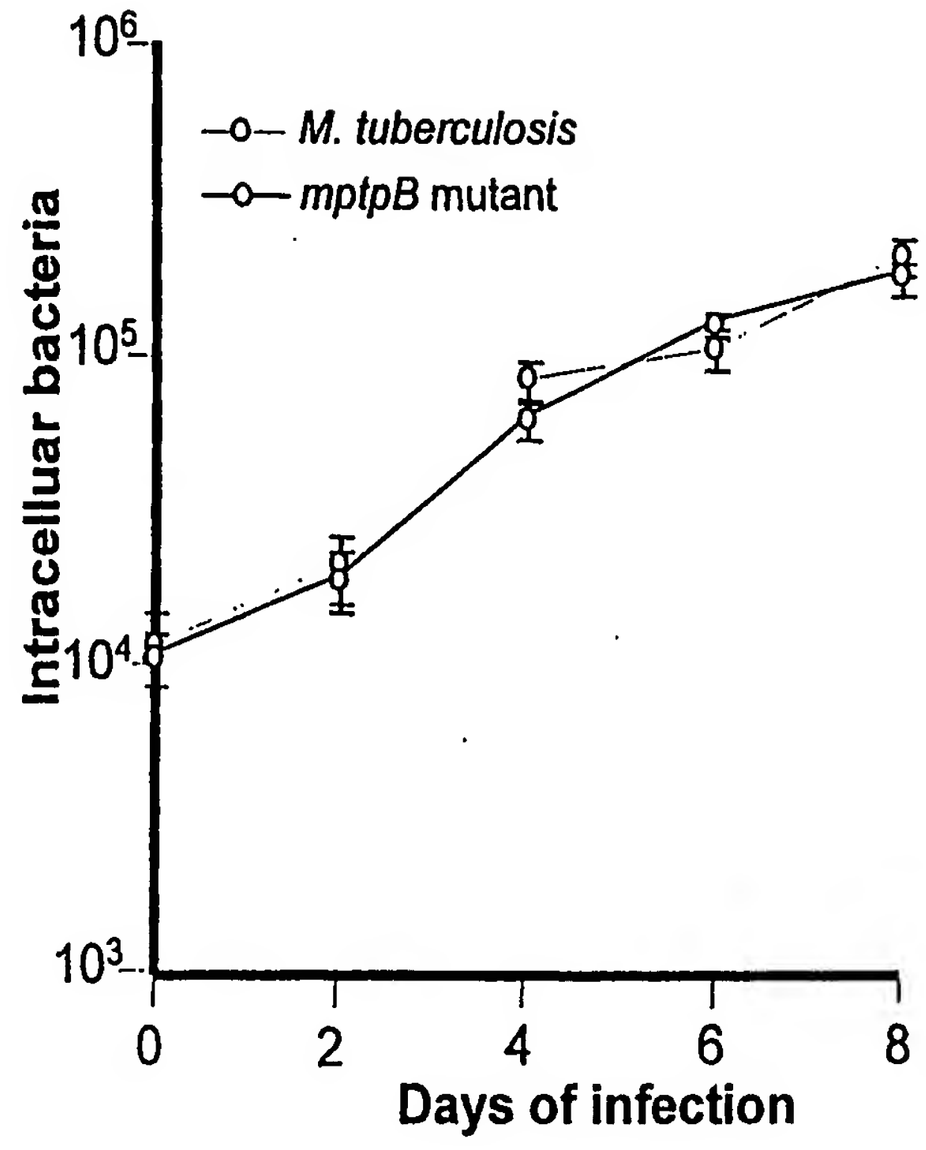

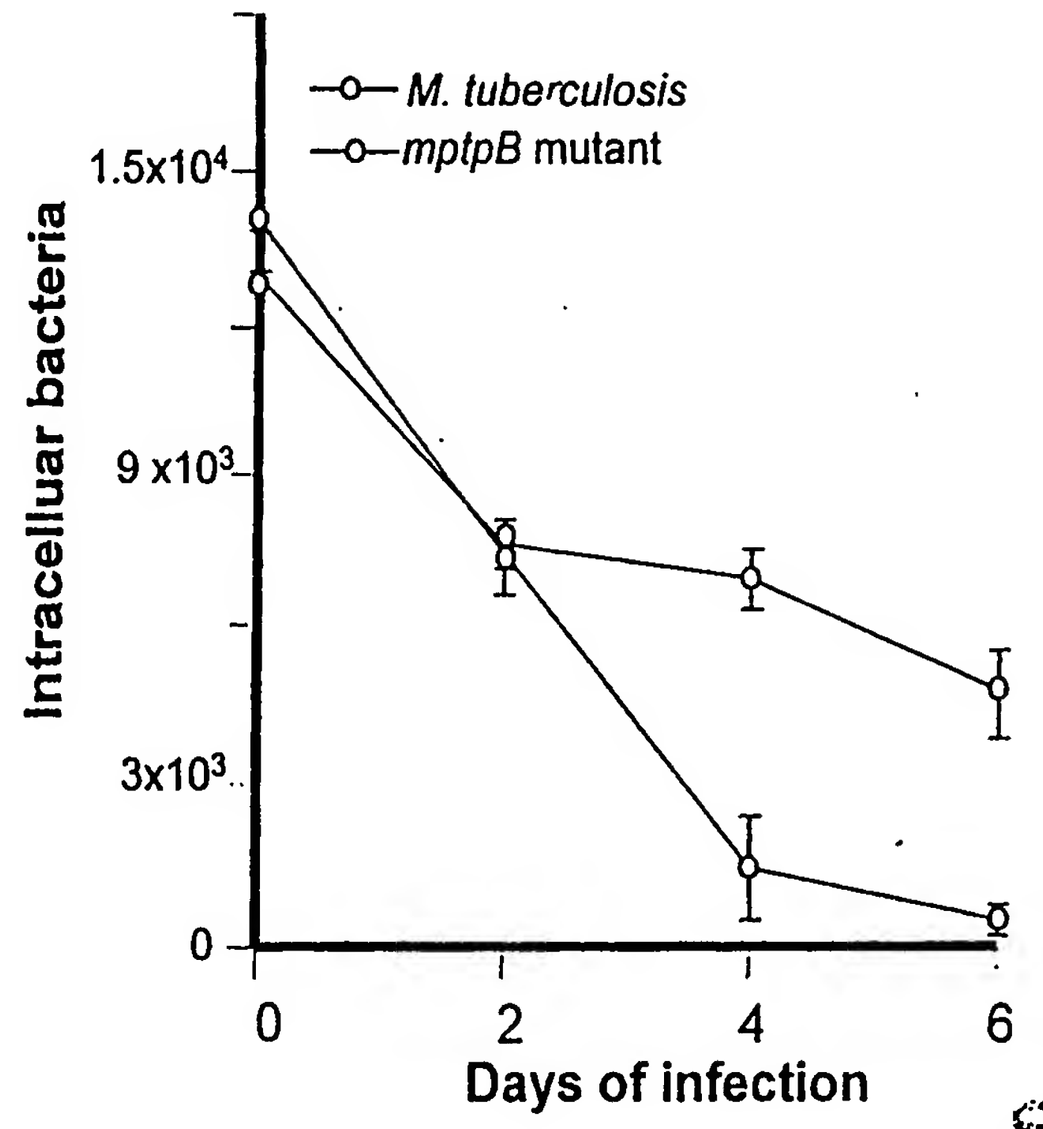

Fig. 8

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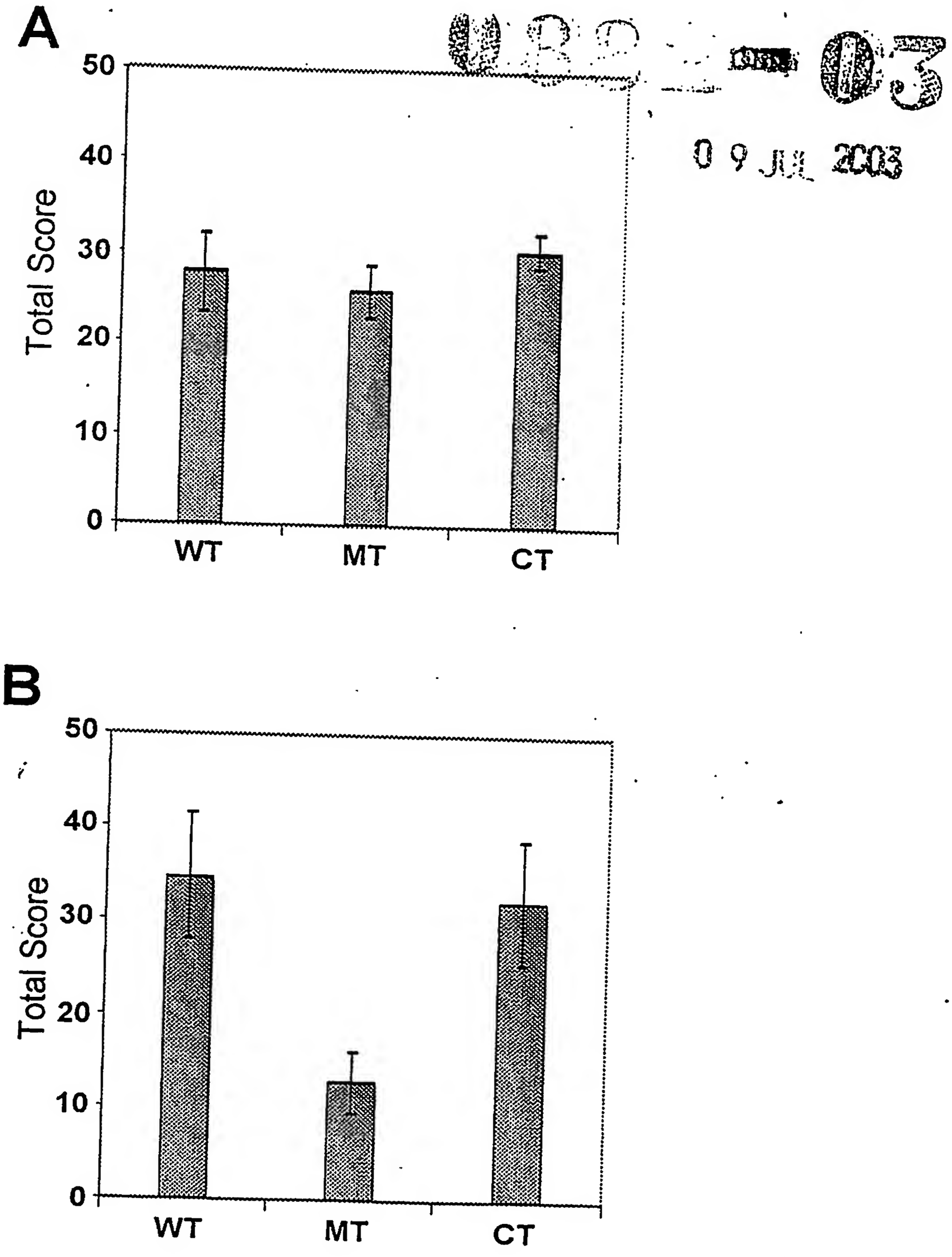

Fig.9

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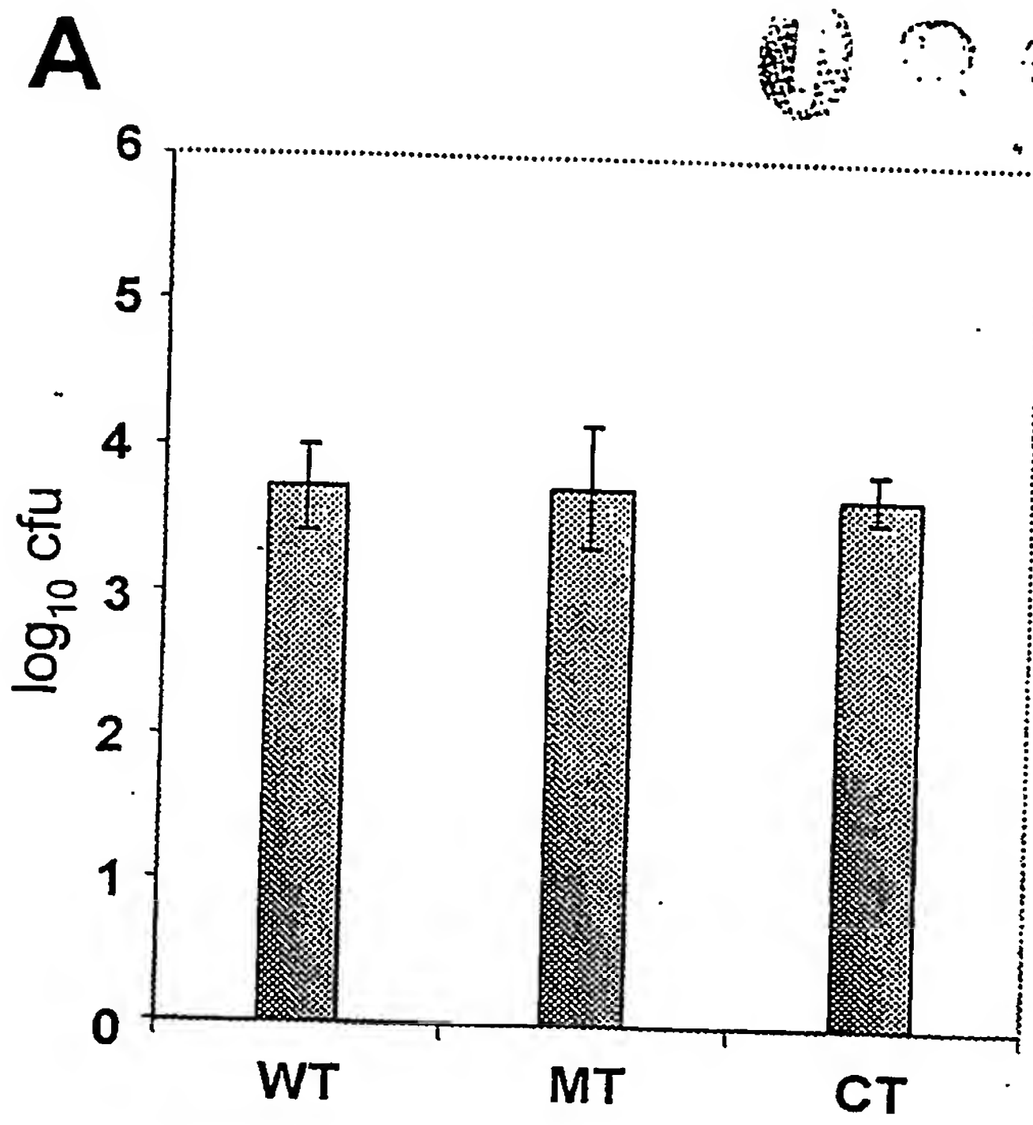
INDIAN COUNCIL OF MEDICAL RESEARCH
IP :NO

TOTAL SHEETS 12
SHEET NO. 10/12



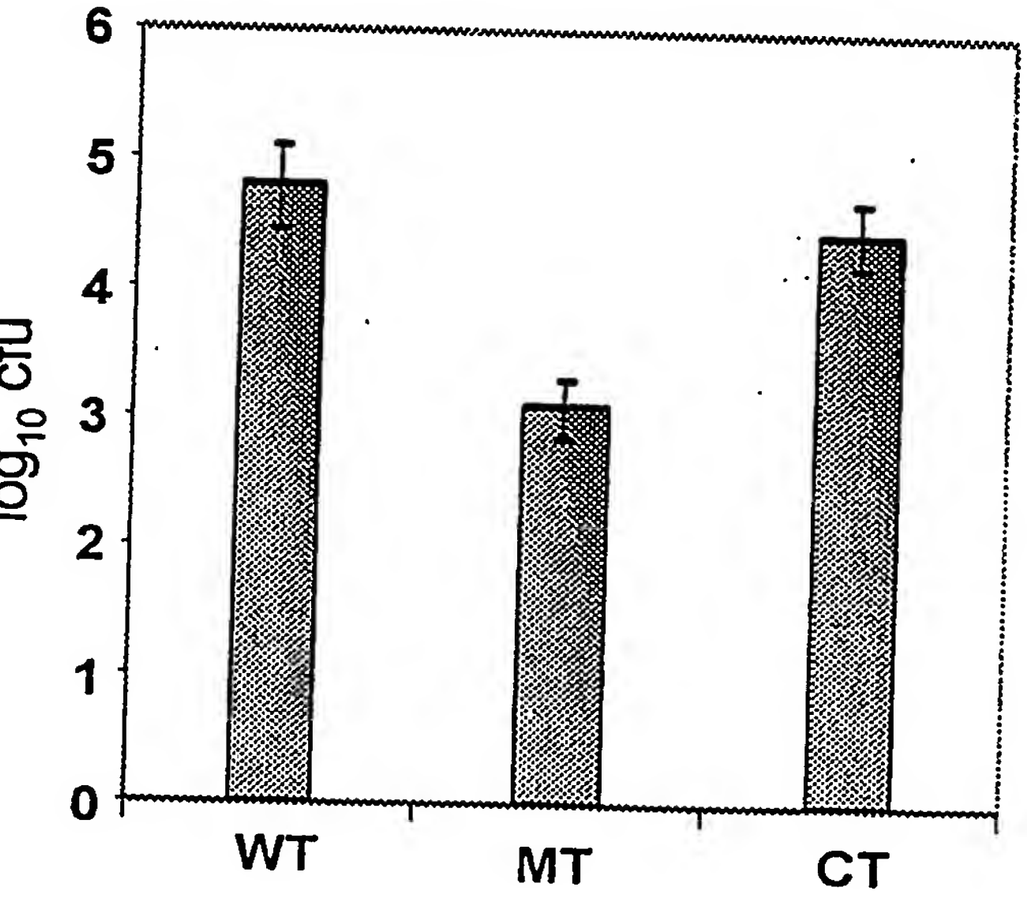


Fig. 10

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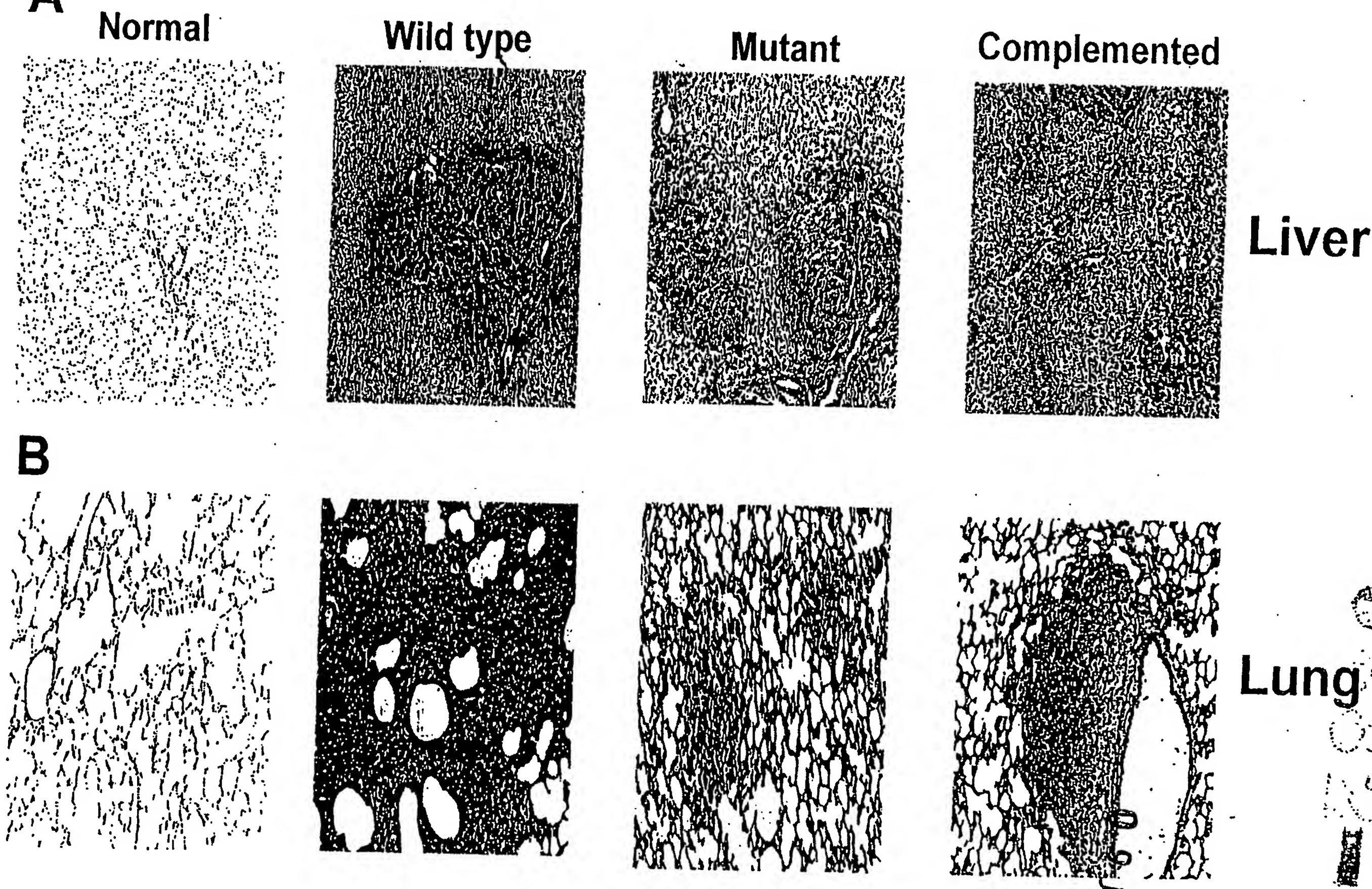

Fig. 11

IP .NO

TOTAL SHEETS 12

SHEET NO. 12/12

A

Normal

Wild type

Mutant

Complemented

Liver

B

Lung

Fig. 12

Rajeshwari

882/DEL/03

- 9 JUL 2004

FORM 2

THE PATENTS ACT, 1970
(39 of 1970)

COMPLETE SPECIFICATION

[See Section 10; rule 13]

MUTANTS OF MYCOBACTERIA AND PROCESS THEREOF

DUPLICATE

We, **INDIAN COUNCIL OF MEDICAL RESEARCH**, an Indian Registered body incorporated under the Registration of Societies Act (Act XXI of 1860) and having their Registered Office at: V. Ramalingaswami Bhawan, Ansari Nagar, Post Box 4911, New Delhi 110 029, India and **UNIVERSITY OF DELHI**, University of Delhi South Campus, Benito Juarez Road, New Delhi 110 021, India

The following specification particularly describes and ascertains the nature of this invention and the manner in which it is to be performed.

MUTANTS OF MYCOBACTERIA AND PROCESS THEREOF

Field of the invention:

The present invention provides an attenuated mutant *Mycobacterium* strain wherein the mutant strain is incapable of expressing the active tyrosine phosphatase and is impaired in its ability to survive in activated macrophages and animals. The invention also provides a method for developing a mutant *Mycobacterium* strain with modified tyrosine phosphatase gene in its genome. The invention also provides a method to assess the role of tyrosine phosphatase in virulence and pathogenesis of mycobacteria and identifies these as potential targets for developing new anti-tubercular drugs.

Background of the invention:

One-third of world's population is infected with *M. tuberculosis* asymptomatically. Eight million new cases of active diseases develop each year & three million people succumb to this disease every year (Dye *et al.*, 1999). With the advent of HIV & emergence of multidrug resistant strains of *M. tuberculosis*, the problem has increased manifold (Horsburgh, 1991; Barnes *et al.*, 1991 and Bloch *et al.*, 1994). The current treatment of disease usually involves combination chemotherapy based on isoniazid, pyrazinamide, rifampicin & ethambutol. In general, 6 months long course is required for effective treatment, which often results in poor compliance on the part of patients, who stop drug intake as soon as they begin to feel better. This leads to development of drug resistant forms of bacilli, which are able to survive routine drug therapy. Multidrug resistant tuberculosis (MDR-TB) is defined as a disease due to tubercle bacilli resistant to at least isoniazid and rifampicin, the two most powerful anti-tubercular drugs. Such a precarious scenario demands development of new drugs that can act on new targets and can be effective in relatively shorter periods so that the patients do not develop resistance to these drugs. The present invention can lead to the development of such target specific anti-tubercular drugs useful for short-term theraples.

Sequence analysis of various prokaryotes has shown the presence of eukaryotic like serine/threonine and tyrosine phosphatases in bacterial pathogens. In various pathogenic bacteria like *Yersinia pseudotuberculosis*, *Salmonella typhimurium* and enteropathogenic *E.coli* tyrosine phosphatases have been shown to act as major virulence determinants (Guan and Dixon., 1990; Galyov *et al.*, 1993 and Kaniga *et al.*, 1996)

YopH, one of the PTPases, is encoded by the yersiniae virulence plasmid and has been identified as an essential virulence factor (Bliska *et al.*, 1991). YopH comprises of several domains including amino terminal sequences involved in secretion, translocation and chaperone binding; a central proline rich SH3 – binding domain and a carboxyl terminal catalytic domain that is homologous to a domain in the eukaryotic PTPases (Sory *et al.*, 1995). It is postulated that YopH disrupts a general phagocytic mechanism as both Fc receptor and complement mediated phagocytosis is inhibited by YopH. (Ruckdeschel *et al.*, 1996 and Fallman *et al.*, 1995). Two of the YopH substrates, $p130^{cas}$ and paxillin are proteins involved in connecting integrins to the actin cytoskeleton and the third one is a tyrosine kinase (Persson *et al.*, 1997 and Black *et al.*, 1997). The possible explanation for the role of YopH protein is that it inhibits uptake of bacteria mediated by the interaction of the bacterial outer membrane protein invasin with cellular β1 integrin. According to this model, invasin binding stimulates tyrosine phosphorylation of cellular targets, leading to cytoskeletal rearrangements and bacterial uptake. YopH dephosphorylates the protein required for this activity. Recent studies have shown that YopH also inhibits Akt pathway and phosphatidylinositol 3 – kinase dependent secretion of interleukin 2 in macrophages (Sauvonnet *et al.*, 2002).

S. typhimurium encodes a tyrosine phosphatase, SptP comprised of modular domains. The amino-terminus of SptP exhibits sequence homology to the Exotoxin S from *P. aeruginosa* and YopE from *Yersinia* spp.. Exotoxin S is an ADP ribosyl transferase that has been implicated in *P. aeruginosa* in the induction of host cell injury and is known to be a virulence factor of *P. aeruginosa*. The carboxyl terminus of SptP showed homology to the eukaryotic like protein tyrosine phosphatases. The carboxyl terminus of SptP protein is homologous to YopH and the catalytic domain of the eukaryotic PTPase. The cysteine residue at position 481 is essential for its catalytic activity as mutation of this conserved cysteine residue abolishes the phosphatase activity (Kaniga *et al.*, 1996). Kaniga *et al* showed that *sptP* mutants are defective in the colonization of spleens of orally infected BALB/c mice. SptP has been shown to possess an *in vitro* GTPase activating protein (GAP) activity towards two host GTP binding proteins, Rac-1 and Cdc42 that play an important role in the cytoskeletal dynamics (Fu and Galan, 1999). It has been suggested that the GAP activity of SptP could down regulate signaling through Cdc42 and Rac that could rebuild the actin cytoskeleton after Salmonella entry. Fu and Galan have shown that microinjection of

purified GST-SptP into cultured cells results in the disruption of actin cytoskeleton and the disappearance of stress fibers (Fu and Galan, 1999).

Prior art:

Allelic exchange by homologous recombination is a powerful tool to study gene functions, identification of virulence factors and development of auxotrophic mutants. "Gene knockout" technique involves the replacement of a wild type gene with it's non-functional counterpart. Such targeted mutations are widely used to study gene functions in mammalian, eukaryotic and bacterial cells (Guilhot *et al.*, 1992; Myers *et al.*, 1994; Reyrat *et al.*, 1995; Baulard *et al.*, 1996; Balsubramaninan *et al.*, 1996;; Azad *et al.*, 1996 ; Azad *et al.*, 1997; Hinds *et al.*, 1999; parish *et al.*, 1999;; Pelicic *et al.*, 1997; Bardarov *et al.*, 1997 and Raynaud *et al.*, 2002).

Sequence analysis of *M. tuberculosis* genome revealed the presence of 11 serine/threonine kinases and two tyrosine phosphatases (Cole *et al.*, 1998). Both genes having sequence homology with known tyrosine phosphatases were PCR amplified by using gene specific primers and *M. tuberculosis* genomic DNA, cloned in a prokaryotic expression vector, pGEX5x-3 and purified from *E. coli* as GST fusion proteins (Koul *et al.*, 2000). The GST fusion proteins were able to dephosphorylate the phospho-tyrosine residue of myelin basic protein but were unable to dephosphorylate phospho-serine and phospho-threonine residues of myelin basic protein. Site directed mutagenesis of cysteine residues in the catalytic motif (Cys11 in the case of MptpA and Cys160 in the case of MptpB) abolished the enzymatic activity (Koul *et al.*, 2000). By Southern blot analysis, it was revealed that *mptpA* is present in fast growing as well as slow growing species of mycobacteria. However, while the mptpB was present in slow growers it was found to be absent in *M. smegmatis*, a fast growing species. (Koul *et al.*, 2000). The present invention was undertaken since the role of tyrosine phosphatase in the virulence and pathogenesis of mycobacterium was not known.

Objects of the invention:

The main objective of the present invention is to develop a mycobacterium strain with a modified tyrosine phosphatase gene in its genome, wherein the mutant *Mycobacterium* strain is incapable of expressing the active tyrosine phosphatase. The *Mycobacterium* species is selected from a group consisting of *M. tuberculosis* and *M. bovis*.

Another object of the present invention is to provide a method for assessing the role of tyrosine phosphatase in the virulence and pathogenesis of *Mycobacterium* in particular *M. tuberculosis*.

Another object of the present invention is to develop a mutant strain of *M. tuberculosis*, which is devoid of the tyrosine phosphatase activity associated with MptpA.

Another object of the present invention is to develop a mutant strain of *M. tuberculosis*, which is devoid of the tyrosine phosphatase activity associated with MptpB.

Still another object of the present invention is to construct a recombinant vector, wherein the recombinant vector carries the *mptpA* gene along with its flanking regions and the internal region of *mptpA* has been substituted by gene conferring resistance to hygromycin.

Still another object of the invention is to insert a second antibiotic resistance marker in the vector backbone particularly kanamycin resistance marker to obtain recombinant vector, pAKΔA.

Another object of the present invention is to construct a recombinant vector, wherein the recombinant vector carries the *mptpB* gene along with its flanking regions and the internal region of *mptpB* has been substituted by gene conferring resistance to hygromycin.

Still another object of the invention is to insert a second antibiotic resistance marker in the vector backbone particularly kanamycin resistance marker to obtain recombinant vector, pBKΔB.

Another object of the invention is to modify the *mptpA* in the genome of *Mycobacterium* strain by homologous recombination using alkali denatured vector, pAKΔA.

Another object of the present invention is to confirm by Southern blot and immuno blot analysis that gene encoding *mptpA* is modified in the genome of *mptpA* mutant *Mycobacterium* strain.

Another object of the present invention is to assess the role of MptpA in the survival of *mycobacterium* in activated macrophages.

Another object of the present invention is to assess the role of MptpA in the survival of mycobacteria in animals, where MptpA can be a potential target for developing new anti-tubercular drugs.

Another object of the invention is to modify *mptpB* in the genome of *Mycobacterium* by homologous recombination using U.V. irradiated vector, pBKΔB.

Another object of the present invention is to confirm by Southern blot and immuno blot analysis that gene encoding *mptpB* is modified in the genome of *mptpB* mutant strain.

Another object of the present invention is to assess the role of MptpB in the survival of m*ycobacterium* in activated macrophages.

Another object of the present invention is to assess the role of MptpB in the survival of mycobacteria in animals, where MptpB can be a potential target for developing new anti-tubercular drugs.

Summary of the invention:

The present invention relates to an attenuated mutant *Mycobacterium* strain having modified tyrosine phosphatase gene wherein the said mutant is incapable of expressing the active tyrosine phosphatase. The invention provides in particular mutant strains of *Mycobacterium tuberculosis* and *Mycobacterium bovis*.

The present invention relates to two tyrosine phosphatase genes *mptpA* and *mptpB* and the role of protein tyrosine phosphatases in the virulence and pathogenesis of *Mycobacterium*.

The present invention also relates to two mycobacterial tyrosine phosphatases (MptpA and MptpB) as potential targets for developing new anti-tubercular drugs.

Further, the invention provides a method for developing an attenuated mutant strain of *Mycobacterium* wherein the tyrosine phosphatase gene is modified in its genome and the said mutant strain is incapable of expressing the active product of tyrosine phophatase gene

Further, the present invention provides a recombinant vector comprising the modified tyrosine phosphatase gene (*mptpA* or *mptpB*).

Further, the recombinant vector contains a selectable marker present within the *mptpA* or *mptpB* gene that may be useful for selection of primary recombinant mycobacteria.

Further, a second antibiotic resistance marker is inserted in the vector backbone to obtain the recombinant vector pAKΔA or pBKΔB.

Further, the recombinant vector may be used to develop mutant strain of *Mycobacterium* wherein homologous recombination may be used to replace active tyrosine phosphatase gene from the wild type strain of *Mycobacterium* by a double cross-over event with a modified tyrosine phosphatase gene.

Further, the mutant strain of *Mycobacterium* may be selected based on the presence of antibiotic resistance marker within the modified tyrosine phosphatase gene.

Further, the invention can be used to develop mutant strains of *Mycobacterium* particularly *Mycobacterium tuberculosis* and *Mycobacterium bovis.*

Further, the invention provides a method for assessing of the role of tyrosine phosphatase in the virulence and pathogenesis of *Mycobacterium,* particularly *Mycobacterium tuberculosis.* Further, the mutant strain of *Mycobacterium* having modified tyrosine phosphatase show reduced survival in the activated macrophages and animals.

Brief description of the accompanying drawings:

Figure 1:

(A) **Southern Blot analysis of the wild type (WT) and *mptpA* mutant (MT1 and MT2) strains of *M. tuberculosis*.**

Genomic DNAs (3µg) from the wild type (WT) and *mptpA* mutant strain (MT1 and MT2) of *M. tuberculosis* were digested with *Not* I, separated on 1.2% agarose gel, transferred to Hybond N membrane and probed with ^{32}P labeled *mptpA* DNA fragment. The size of the DNA standards are shown on the left side of the gel and the size of hybridizing fragment is shown on the right side of the gel.

(B) **Southern Blot analysis of the wild type (WT) and *mptpA* mutant (MT1 and MT2) strains of *M. tuberculosis*.**

Genomic DNAs (3µg) from the wild type (WT) and *mptpA* mutant strain (MT1 and MT2) of *M. tuberculosis* were digested with *Pvu* II, separated on 1.2% agarose gel, transferred to Hybond N membrane and probed with ^{32}P labeled *mptpA* DNA fragment. The size of the DNA standards are shown on the left side of the gel and the size of hybridizing fragment is shown on the right side of the gel.

(C) **Immunoblot analysis of expression of MptpA in the wild type (WT) and *mptpA* mutant (MT1 and MT2) strains of *M. tuberculosis*.**

Analysis of expression of MptpA in the wild type and *mptpA* mutant strain of *M. tuberculosis* by immunoblotting. The strains were grown in 7H9 media to mid-log phase. Equal amounts of whole cell lysate protein (40µg) was resolved on 12.5% SDS-PAGE, transferred to Hybond C Extra membrane and expression of MptpA was analysed by using polyclonal sera raised against MptpA in rabbits.

Figure 2: Survival of the wild type and *mptpA* mutant strains of *M. tuberculosis* in resting and activated macrophages.

The mouse macrophage cell line J774A.1 was infected with the wild type and *mptpA* mutant strain of *M. tuberculosis* separately at an MOI of 1:10 (macrophage: bacilli). At different time points post-infection (day 0, 2, 4, 6 and 8), macrophages were lysed and the number of intracellular mycobacteria was assessed by plating on 7H10 plates (A – in resting macrophages, B – in activated macrophages). The experiments were carried out twice in duplicates and data is depicted as mean of all four values ± S.E.

Figure 3: Bacterial load in spleens of animals infected subcutaneously with 5 x 10^7 cfu of either the wild type (WT) or *mptpA* mutant (MT) of *M. tuberculosis* and euthanised at 3 weeks (A) and 6 weeks (B) post – infection.

Spleens were homogenized in 5 ml of distilled water and ten- fold serial dilutions of the spleen homogenates were plated in duplicates on LJ slopes. Splenic bacillary load of animals euthanised at 3 weeks (A) and 6 weeks (B) post- infection was determined, converted to $\log_{10}$ cfu and depicted as mean ± S.E on y- axis. Various mycobacterial strains are depicted on the x – axis.

Figure 4: Bacterial load in lungs of animals infected subcutaneously with 5 x 10^7 cfu of either the wild type (WT) or *mptpA* mutant (MT) of *M. tuberculosis* and euthanised at 3 weeks (A) and 6 weeks (B) post – infection.

A portion of lungs were homogenized in 5 ml of distilled water and ten- fold serial dilutions of the lung homogenates were plated in duplicates on LJ slopes. Lung bacillary load of animals euthanised at 3 weeks (A) and 6 weeks (B) post- infection was determined, converted to $\log_{10}$ cfu and depicted as mean ± S.E on y- axis. Various mycobacterial strains are depicted on the x – axis.

Figure 5: Histopathology of liver and lung from guinea pigs infected subcutaneously with 5 x 10^7 cfu of either the wild type (WT) or *mptpA* mutant (MT) of *M. tuberculosis* and euthanised 3 weeks post- infection.

Portions of liver and lungs were removed under aseptical conditions and fixed in 10% formalin. Five-micron sections of tissues were stained with haematoxylin and eosin and subjected to histopathological analysis at a magnification of 10X. Representative sections of liver (A) and lung (B) from all the three groups of animals are shown. Sections of liver and lung from uninfected guinea pig were used as reference for normal tissue histology.

Figure 6: Histopathology of liver and lung from guinea pigs infected subcutaneously with 5 x 10^7 cfu of either the wild type (WT) or *mptpA* mutant (MT) of *M. tuberculosis* and euthanised at 6 weeks post - infection.

Sections (5μm) of liver and lung from animals infected with the wild type, *mptpA* mutant and complemented strains of *M. tuberculosis* were fixed, processed, stained with haematoxylin and eosin and observed under microscope at a magnification of 10X. Representative sections of liver (A) and lung (B) from all the three groups of animals are shown. Sections of liver and lung from uninfected guinea pig were used as reference for normal tissue histology.

Figure 7: (A) Southern blot analysis of the wild type and *mptpB* mutant strains (MT1, MT2 and MT3) of *M. tuberculosis*.

Genomic DNAs (3μg) from wild type (WT) and *mptpB* mutant strain (MT1, MT2 and MT3) of *M. tuberculosis* was digested with *Not* I, separated on 1.2% agarose gel, transferred to Hybond N membrane and probed with ^{32}P labeled *mptpB* DNA fragment. The size of DNA standards are shown on the left side of the gel and size of hybridizing band on the right side of the gel.

(B) Immunoblot analysis of the expression of MptpB in wild type (WT) and *mptpB* mutant (MT1, MT2 and MT3) strains of *M. tuberculosis*.

Analysis of the expression of MptpB in wild type and *mptpB* mutant strain of *M. tuberculosis* by immunoblotting. The strains were grown in 7H9 media to mid-log phase. Equal amounts of whole cell lysate protein (40μg) was resolved on 12.5% SDS-PAGE, transferred to Hybond C Extra membrane, the blot was probed for the expression of MptpB using polyclonal sera raised against MptpB in rabbits.

Figure 8: Survival of wild type and *mptpB* mutant strains of *M. tuberculosis* in macrophages.

The mouse macrophage cell line J774A.1 was infected separately with wild type and *mptpB* mutant strain of *M. tuberculosis* at an MOI of 1:10 (macrophage: bacilli). At different time points post-infection (day 0, 2, 4, 6 and 8), macrophages were lysed and the number of intracellular mycobacteria was assessed by plating on MB7H10 plates (A – in resting macrophages, B – in activated macrophages). The experiments were carried out twice in duplicates and data is depicted as mean of all four values ± S.E.

Figure 9: Total post mortem score of guinea pigs infected with 5 x 10^5 cfu of wild type (WT), *mptpB* mutant (MT) and complemented strain (CT) and euthanised at 3 weeks (A) and 6 weeks (B) post – infection.

At the time of sacrifice, depending on the magnitude of pathological damage in spleen, liver, lung, lymph nodes and sites of injection, scores were assigned to each organ as described by Mitchison. Total score for each animal was obtained by totaling up the scores obtained for individual organs and is depicted as mean ± S.E on y - axis. Various mycobacterial strains are depicted on x - axis.

Figure 10: Bacterial load in spleens of guinea pigs infected with 5 x 10^5 cfu of either wild type (WT), *mptpB* mutant (MT) or complemented strain (CT) of *M. tuberculosis* and euthanised 3 weeks (A) and 6 weeks (B) post – infection.

Spleens were homogenized in 5 ml of distilled water and ten- fold serial dilutions of the spleen homogenates were plated in duplicates on LJ slopes. Splenic bacillary load of animals euthanised 3 weeks (A) and 6 weeks (B) post- infection was determined, converted to $\log_{10}$ cfu and depicted as mean ± S.E on y- axis. Various mycobacterial strains are depicted on the x – axis.

Figure 11: Histopathology of liver and lung from guinea pigs infected with 5 x 10^5 cfu of either wild type, *mptpB* mutant or complemented strain of *M. tuberculosis* and euthanised at 3 weeks post- infection.

Portions of liver and lungs were removed under aseptical conditions and fixed in 10% formalin. Five-micron sections of tissues were stained with haematoxylin and eosin and subjected to histopathological analysis at a magnification of 10X. Representative sections of liver (A) and lung (B) from all the three groups of animals are shown. Sections of liver and lung from uninfected guinea pig were used as reference for normal tissue histology.

Figure 12: Histopathology of liver and lung from guinea pigs infected with 5 x 10^5 cfu of either wild type, *mptpB* mutant or complemented strain of *M. tuberculosis* and euthanised at 6 weeks post - infection.

Sections (5μm) of liver and lung from animals infected with wild type, *mptpB* mutant and complemented strains of *M. tuberculosis* were fixed, processed, stained with haematoxylin and eosin and observed under microscope at a magnification of 10X. Representative sections, with an inset of high magnification (20X), of liver (A) and lung (B) from all the three groups of animals are shown. Sections of liver and lung from uninfected guinea pig were used as reference for normal tissue histology.

Detailed description of the invention:

The present invention provides a *Mycobacterium* strain with a modified tyrosine phosphatase gene in its genome, wherein the said *Mycobacterium* strain is incapable

of expressing the active tyrosine phosphatase gene. Further the *Mycobacterium* species is selected from a group consisting of *M. tuberculosis* and *M. bovis*.

The invention provides a *Mycobacterium* strain wherein the modified tyrosine phosphatase gene is either modified *mptpA* or *mptpB* gene. The modified *mptpA* gene is as shown in SEQ ID NO : 15. and the modified *mptpB* gene is as shown in SEQ ID NO : 16.

The invention further provides a recombinant vector comprising the modified *mptpA* or *mptpB* gene. Further, the recombinant vector constructed is either pAKΔA or pBKΔB.

Another aspect of the invention relates to a recombinant vector, wherein the nucleotide sequence of *mptpA* gene is as shown in **SEQ ID NO: 11** is modified. Further, the invention relates to a recombinant vector, wherein the nucleotide sequence of *mptpB* gene is as shown in **SEQ ID NO: 12** is modified.

The invention provides the recombinant vector, wherein the *mptpA* or *mptpB* gene is modified by insertion, deletion, mutation or substitution.

Further, the invention specifically provides a recombinant vector, wherein the *mptpA* or *mptpB* gene is modified by substituting an internal region of the *mptpA or mptpB* gene by an antibiotic resistance marker gene which can be used for selection.

Another aspect of the invention provides a recombinant vector, wherein the antibiotic resistance marker gene imparts resistance to either hygromycin or chloramphenicol preferably to hygromycin.

Further the present invention provides a recombinant vector containing a second antibiotic marker gene for kanamycin resistance in the backbone of the said recombinant vector.

The invention provides both the wild type nucleic acid sequences and the modified forms of the tyrosine phosphatase genes. The invention provides nucleotide sequence of the *mptpA* gene encoding the mycobacterial tyrosine phosphatase A as shown in SEQ ID NO : 11 and modified *mptpA* gene as shown in SEQ ID NO: 15. The invention provides nucleotide sequence of the *mptpB* gene encoding the mycobacterial tyrosine phosphatase B as shown in SEQ ID NO : 12 and modified *mptpB* gene as shown in SEQ ID NO: 16.

Another embodiment of the invention is for a method of developing a mutant *Mycobacterium* strain with a modified tyrosine phosphatase gene in its genome comprising the following steps:

a. extracting genomic DNA from *Mycobacterium* strain,

b. amplifying the tyrosine phosphatase gene along with the flanking sequences using specific primers from the genomic DNA of step (a) to obtain a DNA fragment,

c. characterizing the fragment of step (b),

d. cloning the fragment of step (b) in a non-replicative vector,

e. modifying the fragment in the non-replicative vector of step (d),

f. inserting an antibiotic resistance marker gene within the fragment of step (e) to obtain a non-replicative vector containing a modified tyrosine phosphatase gene,

g. cloning of a second antibiotic resistance marker gene in the backbone of the non-replicative vector of step (f), to obtain a recombinant vector,

h. introducing the recombinant vector of step (g) into *Mycobacterium strains*,

i. selecting for primary recombinant *Mycobacterium strains* using first antibiotic selection marker gene,

j. culturing the primary recombinant *Mycobacterium strains* of step (i) harboring the first antibiotic resistance marker gene,

k. selecting the secondary recombinant *Mycobacterium strains* of step (j) that is sensitive to the second antibiotic resistance gene present in the vector backbone,

l. culturing the secondary recombinant *Mycobacterium strains* of step (k), wherein the said recombinant *Mycobacterium* strain harboring the modified tyrosine phosphatase gene which shows defective growth in activated macrophages and animals.

Further, the invention provides a method wherein, the *Mycobacterium* species is selected from a group consisting of *M. tuberculosis* and *M. bovis*

Another aspect of the invention provides a method wherein the specific primers are selected from a group comprising of SEQ ID NO: 1 to 4 for amplification of *mptpA* along with its flanking regions and SEQ ID NO : 5 to 8 for amplification of *mptpB* along with its flanking regions.

The invention further provides a method, wherein the *mptpA* or *mptpB* gene is modified by insertion, deletion, mutation or substitution specifically by substituting an internal region of the *mptpA or mptpB* gene by an antibiotic resistance marker gene preferably hygromycin resistance gene.

The invention provides a method, wherein in the second antibiotic marker gene imparting resistance to kanamycin is inserted in the recombinant vector backbone.

Yet another aspect of the invention is to modify tyrosine phosphatase gene in the genome of *Mycobacterium* using either recombinant vector pAKΔA or pBKΔB.

The present invention further provides a method wherein homologous recombination may be used to replace the active tyrosine phosphatase gene of mycobacteria by a double cross over event with a modified gene to develop a mutant *Mycobacterium* strain.

Another embodiment of the invention is to assess the role of MptpA and MptpB in the virulence and pathogenesis of mycobacteria in activated macrophages and animals.

Further the invention shows that the mutant *Mycobacterium* strains are attenuated and impaired in their ability to survive in activated macrophages and animals.

Further the invention relates to two tyrosine phosphatases MptpA and MptpB of mycobacteria which are potential targets for developing anti-tubercular drugs.

(A) Construction of recombinant vector, pAKΔA.

The mutant strain lacking tyrosine phosphatases associated with either MptpA was employed to understand the role of these proteins in the survival of *M. tuberculosis* in murine macrophages and in the ability of the mutants to cause disease in guinea pigs.

The wild type tyrosine phosphatase gene was modified to develop a mutant strain of *Mycobacterium*. The genome of *Mycobacterium* encodes for two tyrosine phosphatase, MptpA and MptpB. The genomic DNA from mycobacterium strain was extracted by CTAB standard methods as given in Example 3. The gene for *mptpA* was amplified from the genome using specific primers as shown in Table 1 and also given in Example 4.

Based on the genome sequence of *M. tuberculosis*, the primers were designed to amplify *mptpA* (SEQ ID NO: 11) along with its upstream and downstream flanking regions. A DNA fragment carrying 1135 bp upstream to the *mptpA* ORF along with the initial 156 bp of *mptpA* ORF was PCR amplified by using *M. tuberculosis* DNA as template and primer A (SEQ ID NO: 1) and primer B (SEQ ID NO: 2) carrying a *Nde* I site at the 5'end. The amplicon was end-repaired and cloned into *Eco*R V digested vector pLitmus-38 resulting in vector pLitA1. The ligation was transformed into *E. coli* as given in Example 5. The plasmids were isolated from the recombinants as given in Example 6. The recombinants were analysed by restriction enzyme digestion as given in Example 7.

Another DNA fragment carrying 167 bp of *mptpA* ORF corresponding to the C-terminal region of MptpA along with 1240 bp downstream to the *mptpA* ORF was PCR amplified by using gene specific primers, primer C (SEQ ID NO: 3) carrying a *Nde* I site at the 5' end and primer D (SEQ ID NO: 4) carrying a *Bsp*H I site at the 5'end. The amplicon was end-repaired and separately cloned into *Eco*R V digested vector pLitmus-38 resulting in vector pLitA2. The vector pLitA1 was digested with *Nde* I and *Sca* I and the larger DNA fragment containing the initial 156 bp of *mptpA* ORF along with 1135 bp upstream to the *mptpA* ORF was gel purified by standard procedure as given in Example 8 and 9. Similarly, vector pLitA2 was digested with *Nde* I and *Sca* I and the smaller DNA fragment containing the 167 bp of *mptpA* ORF corresponding to the C-terminal region of MptpA along with 1240 bp downstream to the *mptpA* ORF was gel purified. The larger fragment obtained by the digestion of pLitA1 and the smaller fragment obtained by the digestion of pLitA2 were then ligated together resulting in pLitΔA. The *mptpA* specific primers primer B (SEQ ID NO: 2) and primer C (SEQ ID NO: 4) were non-overlapping; as a result, the vector pLitΔA contained the coding region of *mptpA* with a deletion of 112 bp from the central region of ORF. The vector pLitΔA comprises nucleotide sequence as shown in SEQ ID NO: 13. The insert sequence in the vector was characterized by sequencing as shown in Example 11.

The vector also carried 1135 bp of upstream and 1240 bp of downstream flanking sequences and a unique *Nde* I site in the ORF of *mptpA* at the deletion site for the cloning of hygromycin resistance gene. The hygromycin resistance gene was excised out from pLit28res-hyg-res as a *Bam*H I – *Xba* I fragment, end-repaired and cloned into *Nde* I digested, end-repaired pLitΔA resulting in pLitΔAH. The insert sequence in the vector was characterized by sequencing as shown in Example 11. The vector pLitΔAH comprises an insert having nucleotide sequence as shown in SEQ ID NO: 15.

A 4.8kb DNA fragment containing *mptpΔA :: hyg*^{r} was excised out from pLitΔAH as a *Spe* I–*Nhe* I fragment and cloned into *Xba* I -digested pJQ200SK (a non-replicative suicide vector) yielding pJQΔA. A second antibiotic resistance marker for kanamycin resistance was inserted in the vector backbone as given below.

The gene conferring resistance to kanamycin was excised out from pSD5 as an *Nhe* I – *Bst*E II fragment, end repaired and cloned into *Sma* I digested pJQΔA resulting in pAKΔA. The recombinant vector pAKΔA provided 1.3 kb and 1.4 kb homologous region on either side of the hygromycin resistant gene for recombination to occur at *mptpA* locus between targeting DNA and the mycobacterial genome.

The recombinant vector pAKΔA comprises the modified *mptpA* gene (SEQ ID NO 15) and a second antibiotic resistance marker in the backbone.

(B) Construction of recombinant vector pBKΔB.

For disruption of *mptpB* of *M. tuberculosis*, vector pBKΔB was constructed. The genomic DNA from mycobacterium strain was extracted by CTAB standard methods as given in Example 3. The gene for *mptpB* (SEQ ID NO : 12) was amplified from the genome using specific primers as shown in Table 1 and also given in Example 4.

Based on the genome sequence of *M. tuberculosis*, the primers were designed to amplify *mptpB* (SEQ ID NO: 12) along with its upstream and downstream flanking regions. For this, a DNA fragment containing 1045 bp upstream to the ORF of *mptpB* along with the initial 356 bp of ORF of *mptpB* was PCR amplified using *M. tuberculosis* DNA as template and primers E (SEQ ID NO: 5) and primer F (SEQ ID NO: 6). The amplicon was end-repaired and cloned into *Eco R* V digested vector pLitmus-38 resulting in vector pLitB1. The ligation was transformed into *E. coli* as given in Example 5. The plasmids were isolated from the recombinants as given in Example 6. The recombinants were analysed by restriction enzyme digestion as given in Example 7.

Another DNA fragment containing 367 bp of *mptpB* ORF corresponding to the C-terminal region of MptpB along with 1140 bp downstream to the ORF of *mptpB* was PCR amplified using gene specific primers primer G (SEQ ID NO: 7) and primer H (SEQ ID NO : 8). The amplicon was end-repaired and separately cloned into *EcoR* V digested vector pLitmus-38 resulting in vector pLitB2. The vector pLitB1 was digested with *Nde* I and *Sca* I and the larger DNA fragment containing the initial 356 bp of *mptpB* ORF along with 1045 bp upstream to the ORF was gel purified using a Qiagen gel extraction kit. Similarly, pLitB2 was digested with *Nde* I and *Sca* I and the smaller DNA fragment containing the 367 bp of *mptpB* ORF corresponding to the C-terminal region of MptpB along with 1140 bp downstream to the ORF was gel purified. The larger fragment obtained by the digestion of pLitB1 and the smaller fragment obtained by the digestion of pLitB2 were then ligated together resulting into pLitΔB. The *mptpB* specific primers primer F (SEQ ID NO : 6) and primer G (SEQ ID NO : 7) were non-overlapping, as a result, the vector pLitΔB contained the coding region of *mptpB* (with a deletion of 108 bp from the central region of ORF) and 1045 bp of upstream and 1140 bp of downstream flanking sequences and a unique *Nde* I site in the ORF of *mptpB* at the deletion site for the cloning of hygromycin resistance gene cassette. The

vector pLitΔB comprises nucleotide sequence as shown in SEQ ID NO : 14. The insert sequence in the vector was characterized by sequencing as shown in Example 11.

The hygromycin resistance gene cassette was excised out from pLit28res-hygres as a *BamH* I – *Xba* I fragment, end-repaired and cloned into *Nde* I digested, end-repaired pLitΔB resulting in pLitΔBH. The insert sequence in the vector was characterized by sequencing as shown in Example 11. The vector pLitΔBH comprised the insert having nucleotide sequence as shown in SEQ ID NO : 16.

A 4.9 kb DNA fragment containing *mptpΔB*::hyg^r was excised out from pLitΔBH as a *Spe* I–*Nhe* I fragment and cloned into *Xba* I -digested pJQ200SK (a non-replicative suicide vector, Pelicic *et al.*, 1996) yielding pJQΔB. A second antibiotic resistance marker for kanamycin resistance was inserted in the vector backbone as given below.

The gene conferring resistance to kanamycin was excised out from pSD5 as an *Nhe* I–*BstE* II fragment, end repaired and cloned into *Sma* I-digested pJQΔB resulting in pBKΔB. The vector pBKΔB provided 1.4 kb and 1.5 kb homologous regions upstream and downstream of the hygromycin resistant gene, respectively, for recombination to occur between targeting DNA and the mycobacterial genome.

The recombinant vector pBKΔB comprises the modified *mptpB* gene (SEQ ID NO: 16) and as second resistance marker in the backbone.

(C) Modification of the *mptpA* in the genome of *Mycobacterium* and its role in the virulence and pathogenesis of *M. tuberculosis*.

In order to evaluate the role of MptpA in the pathogenesis of *M. tuberculosis*, an *mptpA* mutant strain was constructed by using a non-replicative vector pAKΔA having modified *mptpA* sequence as shown in SEQ ID NO : 15. The recombinant vector, pAKΔA carried the coding region of *mptpA* along with it's 1135 bp upstream and 1240 bp downstream flanking sequences of *mptpA*. A portion of the coding region (112 bp) of MptpA was deleted and replaced with gene conferring resistance to hygromycin in pAKΔA. Electroporation of *M. tuberculosis* Erdman with non-replicative vector, pAKΔA and alkali denatured pAKΔA resulted in 39 and 2 hygromycin resistant transformants, respectively on 7H10 plates supplemented with hygromycin (50μg/ml). The details are of electroporation are given in Example 12. The alkali pretreatment is as given in Example 13. All the transformants were PCR positive for hygromycin resistance gene suggesting that plasmid borne *mptpΔA::hyg^r* had integrated into the

mycobacterial genome. Allelic exchange by homologous recombination should result in incorporation of the hygromycin resistance gene but not the vector backbone (carrying kanamycin resistance gene) into the mycobacterial genome. Thus, the transformants were screened for kanamycin resistance gene by PCR using gene specific primers. The transformants obtained upon electroporation of pAKΔA were PCR positive for the kanamycin cassette, where as the two transformants obtained upon electroporation of alkali denatured pAKΔA were PCR-negative for the kanamycin cassette. These results indicated that homologous recombination at *mptpA* locus had occurred in the case of transformants obtained upon electroporation of alkali denatured DNA. Thus, transformants resistant to hygromycin but sensitive to kanamycin were selected to score for homologous recombination event.

The disruption of *mptpA* in the mycobacterial genome was verified by Southern blot analysis using *mptpA* specific DNA probe (SEQ ID NO : 11). The details of the southern blot hybridization and preparation of nucleic acid probes are given in Example 14 and 15). As expected, for allelic exchange event to occur at homologous site, in the lanes corresponding to the two hyg^r kan^s transformants, a single hybridizing fragment 4.1 kb, 2kb longer than that in the wild type strain (2.1kb) was observed. This increase in the size of the band by 2.0 kb in both hyg^r kan^s transformants corresponded to the replacement of 112bp internal fragment of *mptpA* with hygromycin resistance gene (Fig 1A). Immunoblot analysis (as given in Example 10) of whole cell lysate demonstrated that disruption of *mptpA* resulted in lack of expression of MptpA in the mutant strain (Fig. 1B).

To investigate the role of MptpA in the intracellular survival of *M. tuberculosis*, the survival rates of *mptpA* mutant and its parental strain were compared in resting as well as in IFN-γ activated mouse macrophage cell line, J774A.1. The numbers of intracellular surviving bacteria were calculated at days 0,2,4,6 and 8 post-infection. Both parental as well as *mptpA* mutant strain displayed a similar pattern of intracellular growth in resting macrophages. While at the initial time point (day0) bacillary counts were approximately 2×10^4 per well. The bacillary load increased at later time points attaining peak values of 2×10^5 at day 8 post-infection. These results showed that both parental as well as *mptpA* mutant strains of *M. tuberculosis* exhibited comparable capacity of infection and multiplication in resting macrophages (Fig. 2A). However, both the strains differed in their ability to survive in IFN-γ activated macrophages. In activated macrophages approximately 45%, 50% and 70% killing of wild type bacilli was observed, at days 2,4 and 6 post-infection, respectively, in comparison to 70%,

95% and 98% killing of *mptpA* mutant strain at days 2, 4 and 6 post-infection, respectively (Fig. 2B). These observations indicated that disruption of *mptpA* had impaired the ability of *M. tuberculosis* to survive in IFN-γ activated macrophages. The details of *in vitro* studies in macrophages are shown in Example 16.

To determine whether MptpA plays a role in the pathogenesis of *M. tuberculosis*, guinea pigs in groups of 16 animals were infected subcutaneously with 5 x 10^7 cfu of parental, mutant or complemented strain of *M. tuberculosis*. Animals were euthanised 3 weeks and 6 weeks post-infection. At both time points of euthanisation (7 animals per group), number of colony forming units in spleen and lungs were enumerated (represented as $\log_{10}$cfu for each group).

The *mptpA* mutant strain was significantly attenuated for growth in guinea pig model of tuberculosis. At 3 weeks post-infection a 9-fold reduction was observed in the bacillary load in spleens of animals infected with *mptpA* mutant strain ($\log_{10}$ 5.09 ± 0.23) as compared to the parental strain ($\log_{10}$ 5.99 ± .27, Fig. 3A). A similar reduction in cfu was also observed in the lungs of animals infected with *mptpA* mutant strain, ($\log_{10}$ 3.07 ± .13) as compared to ($\log_{10}$ 3.95 ± 0.32) in the lungs of animals infected with the parental strain (Fig. 4A). The differences in the bacterial load in the spleen and lungs of animals infected with *mptpA* mutant strain as compared to the bacterial load of animals infected with parental strain increased from 9 folds to 90 folds at six weeks post-infection. The bacillary load in the animals infected with *mptpA* mutant strain was $\log_{10}$ 4.83 ± 0.43 for spleens and 3.71± 0.30 for lungs, when compared to the bacillary load in animals infected with parental strain 6.73 ± 0.33 for spleens and 5.62 ±0.38 for lungs (Fig. 3B and 4B, respectively). The reduction in the bacillary load in the spleens and lungs of animals infected with *mptpA* mutant strain was found to be statistically significant ($p < 0.002$ in the case of spleens and $p < 0.001$ in the case of lung, respectively).

Sections of liver and lung from various groups were analysed histologically to determine the extent of tissue damage. Fig. 5 depicts the mean percentage of granuloma and cellular composition in liver granuloma of animals at 3 weeks post-infection. At 3 weeks post-infection, the animals infected with the parental strain exhibited 5.4% liver granuloma. The liver granuloma comprised of 10% lymphocytes, 8% macrophages and 82% epitheloid cells. In case of animals infected with the *mptpA* mutant strain, 10% liver granuloma was observed and the granuloma comprised of 21% lymphocytes, 11% macrophages and 68% epitheloid cells (Fig. 5).

In case of lung, no significant difference was observed in the percentage of granulomatous tissue and cellular composition of the granuloma in case of animals infected with various strains. The animals infected with parental strain exhibited 14% lung granuloma and lung granuloma comprised of 30% lymphocytes and 70% macrophages. In case of animals infected with *mptpA* mutant strain 21.5% lung granuloma was observed and the granuloma comprised of 30% lymphocytes and 70% macrophages. Representative sections of liver and lung of animals infected with the parental or *mptpA* mutant strain at 3 weeks post-infection are shown in Fig. 6. The details of guinea pig studies is shown in Example 17.

(D) Modification of the *mptpB* in *M. tuberculosis* and its effect on the pathogenesis of *M. tuberculosis*:

In order to establish whether MptpB plays a role in the pathogenesis of *M. tuberculosis*, a *mptpB* mutant strain of *M. tuberculosis* was constructed by using a non-replicative suicidal vector pBKΔB having a modified *mptpB* sequence as shown in SEQ ID NO: 16. The targeting vector, pBKΔB carried the coding region of *mptpB* along with 1045 bp upstream and 1140 bp downstream flanking sequences. A portion of the coding region (108 bp) of MptpB was deleted and replaced with the gene conferring resistance to hygromycin in pBKΔB. The vector also carried the gene conferring resistance to kanamycin in its backbone as a second antibiotic selection marker for negative screening of allelic exchange events at the homologous site.

Electroporation of *M. tuberculosis* with pBKΔB and U.V. irradiated pBKΔB resulted in 22 and 3 hygromycin resistant transformants, respectively. The details of electroporation are given in Example 12. The U.V. irradiation is as given in Example 13. PCR analysis revealed that all the transformants contained hygromycin cassette indicating that these colonies were not spontaneous resistance mutants and arose from integration of the suicidal vector into the mycobacterial genome. Allelic exchange event by homologous recombination should result in the incorporation of hygromycin resistance gene but not the vector backbone (having kanamycin resistance gene) into the mycobacterial genome. Thus, transformants resistant to hygromycin but sensitive to kanamycin were selected to screen for homologous recombination event. All the transformants obtained on electroporation of untreated DNA were kanamycin resistant while the three transformants obtained on electroporation of U.V. pretreated DNA were sensitive to kanamycin. This suggested that an allelic exchange event at the

homologous site had taken place in the case of these three hyg^r kan^s transformants obtained upon electroporation of U.V. irradiated DNA.

mptpB gene disruption was assessed by hybridization analysis of genomic DNA isolated from the parental *M. tuberculosis* strain and three hyg^{R}kan^s transformants. A DNA fragment containing the entire coding region of *mptpB* (SEQ ID NO : 12) was used as probe as given in Example 15. Southern blot analysis (as given in Example 14) showed presence of a 1.85 kb band in the parental strain whereas a 3.8 kb band was observed in all the three hyg^{R}kan^s transformants as expected upon replacement of 108 bp internal fragment of *mptpB* with hygromycin resistance gene cassette (Fig. 7A). These results indicated that *mptpB* was disrupted in all the three hyg^{R}kan^s transformants. Expression of MptpB was analysed in the mutant strains using polyclonal sera raised against MptpB in rabbit. Western blot analysis (as shown in Example 10) showed absence of MptpB expression in all the three mutant strains (Fig. 7B). The complemented strain was constructed by electroporation of pSD5-*mptpB* into electrocompetent cells of the mutant strain. The electroporation of pSD5-*mptpB* restored the expression of MptpB in the complemented strain (Fig. 7B).

To study the effect of disruption of *mptpB* gene on the intracellular survival of *M. tuberculosis*, resting and IFN-γ activated murine macrophage cells were infected with either the wild type or *mptpB* mutant strain of *M. tuberculosis*. The number of surviving intracellular bacteria was determined on days 0, 2, 4, 6 and 8 post - infection. Both parental as well as the *mptpB* mutant strain displayed a similar pattern of intracellular growth at all time points of study (Fig. 8A). While at the initial time point (day 0) the bacillary counts were approximately 10^4 cfu/well, the bacillary load increased at later time points attaining the peak values of ~ 10^5 cfu at 8 days post - infection. These results showed that both parental as well as the *mptpB* mutant strain exhibited comparable capacity of infection and multiplication in resting mouse macrophages. However, the two strains showed differences in their ability to survive in the activated macrophages. The number of wild type *M. tuberculosis* and *mptpB* mutant was maximum and comparable at the initial time point (~10^4 cfu/well, at day 0). At later time points, a reduction in the number of bacilli was observed in both cases. While the wild type *M. tuberculosis* was reduced to 50% and 28.6% at days 4 and 6 post- infection, respectively, a much sharper decline was noted in the case of *mptpB* mutant which was reduced to 10% and 4% at days 4 and 6 post – infection, respectively (Fig. 8B). These observations indicated that disruption of *mptpB* gene

had impaired the ability of *M. tuberculosis* to survive in IFN-γ activated macrophages. The details of *in vitro* studies in macrophages are shown in Example 16.

To determine whether the disruption of *mptpB* gene would have any effect on the survival of *M. tuberculosis in vivo*, guinea pigs in groups of eight animals were infected subcutaneously with $5x10^5$ cfu of either parental, mutant or the complemented strain of *M. tuberculosis*. Animals were euthanized three weeks and six weeks post-infection. At both time points of euthanization, spleens were homogenized and viable bacilli were enumerated (represented as $\log_{10}$ cfu for each group).

It was observed that at 3 weeks post-infection, the mean total score of the animals infected with mutant strain was 26, which was comparable to the scores in case of animals infected with parental (28) and complemented strain (30, Fig.9A). These results were commensurate with the splenic cfu obtained for various groups on euthanization of animals at 3 weeks post-infection. The bacterial load in the spleen of animals infected with the mutant strain was $\log_{10}$ 3.71, which was comparable to the bacterial load in the spleens of animals infected with parental ($\log_{10}$ 3.73) and complemented strain ($\log_{10}$ 3.68, Fig.10A). However, the total scores of the animals infected with mutant strain at the end of six weeks was significantly lower (12) than the total score of animals infected with parental (35, $p<0.02$) and complemented strain (33, $p<0.02$, Fig.9B). The animals infected with mutant strain exhibited a significant reduction of bacillary load in spleen ($\log_{10}$ 3.07) when compared to bacillary load in spleen of animals infected with parental ($\log_{10}$ 4.77, $p<0.002$) and complemented strain ($\log_{10}$ 4.45, $p<0.003$, Fig. 10B). Thus, an approximately 3-fold reduction in total score and a 50 to 70-fold reduction in the bacillary load in spleens was observed in animals infected with *mptpB* mutant strain in comparison to parental or complemented strains.

Sections of liver and lung from animals in various groups were subjected to histological analysis to determine morphology of the organs, the presence and extent of granuloma and the type and number of infiltrating cells. It was observed that at three weeks there were no significant histological differences in liver and lung of animals infected with either parental, or mutant or complemented strain. At 3 weeks post-infection animals from all 3 groups showed no difference in the extent or composition of granuloma. In case of liver, granuloma consisted mainly of epitheloid cells and lymphocytes, while the lung granuloma comprised mainly of lymphocytes macrophages and a few epitheloid cells (Fig. 11A and 11B). At six weeks post –

infection, in the case of animals infected with wild type and complemented strain, the liver sections showed multiple well-defined granuloma comprising of epitheloid cells and lymphocytes. However, the liver tissue from animals infected with the *mptp*B mutant strain exhibited a distinct qualitative difference with respect to the presence of epitheloid cells with only a few lymphocytes. In case of lung tissues, the animals infected with the wild type and complemented strain showed extensive granulomas comprising of lymphocytes and macrophages. In contrast, the lung tissue from animals infected with the mutant strain showed partly organized granuloma mainly of lymphocytes (Fig. 12A and 12B). The details of guinea pig studies is shown in Example 17.

Statistical analysis

Data are depicted as arithmetic mean ± standard error mean. Data were analyzed for statistical significance using the Student's t test. Differences between the guinea pig groups were considered significant if p values were <0.05.

Brief description of the accompanying table:

Table 1: Sequence listing

SEQ ID NO : 1	Primer A
SEQ ID NO : 2	Primer B
SEQ ID NO : 3	Primer C
SEQ ID NO : 4	Primer D
SEQ ID NO : 5	Primer E
SEQ ID NO : 6	Primer F
SEQ ID NO : 7	Primer G
SEQ ID NO : 8	Primer H
SEQ ID NO : 9	Primer I
SEQ ID NO : 10	Primer J
SEQ ID NO :11	*mptpA gene*
SEQ ID NO : 12	*mptpB gene*
SEQ ID NO : 13	*mptpAL (with flanking sequences)*
SEQ ID NO : 14	*mptpBL (with flanking region)*
SEQ ID NO : 15	Modified *mptpA*

SEQ ID NO : 16	Modified *mptpB*

Examples:

The following methods are listed to illustrate the invention and should not be construed to limit the scope of the invention.

Examples:

Example 1 Source of reagents and chemicals used:

Reagents, chemicals and enzymes including media for growing culture were purchased from standard sources.

Example 2 : Culturing of bacterial strains (*E.coli* and Mycobacteria)

E. coli was grown in either Luria Bertani medium or in 2XYT medium supplemented with either of the antibiotics; ampicillin (50 μg/ml); kanamycin (25 μg/ml); gentamycin (50 μg/ml) or hygromycin (150 μg/ml). *M. tuberculosis* Erdman was grown in Middlebrook 7H9 medium supplemented with 0.5% glycerol, 0.2% Tween-80 and 1X ADC supplement. The cultures were grown with constant shaking at 200 rpm, 37°C. Solid media included LB Agar in case of *E. coli* and 7H10/7H11 media containing 0.5% glycerol, 1XOADC supplement and appropriate antibiotics in case of *M. tuberculosis*.

Example 3: Isolation of genomic DNA from mycobacteria:

Mycobacteria was grown to an A_{600nm} of 2-3 and glycine was added to the culture at a final concentration of 1%. 24 hours after addition of glycine, cells were harvested by centrifugation at 8,000 rpm for 10 minutes at room temperature. The pellet was resuspended in 500 μl of TEG solutions and 50 μl of lysozyme (20 μg/ml) was added. After overnight incubation at 37°C, lysis was carried out by the addition of 100 μl of 10% SDS and 50 μl of Proteinase K (10 mg/ml) followed by incubation at 55°C for 40 minutes. To the cell lysate, 200 μl of NaCl and 160 μl of CTAB was added and the suspension was incubated at 65°C for 10 minutes. The lysate was extracted twice with phenol (pre-equilibrated with Tris-Hcl, pH 8.0) and twice with chloroform. The DNA was precipitated by adding 1/10th volume of 3M sodium acetate and two volumes of chilled ethanol. The DNA pellet was then washed with 70% ethanol and resuspended in 100 μl of autoclaved double distilled water.

Example 4: Polymerase Chain Reaction (PCR):

Amplification of genes by PCR was carried out as per manufacturer's recommendations. All PCR reactions were performed by using Taq/Pfu mix. The

sequences of oligonucleotides used are shown in Table 1. A typical amplification reaction contained 10 ng of template DNA, 1x Taq polymerase buffer, 200 µM dNTPs, and 20 pmoles each of forward and reverse primers, 1.5 mM $MgCl_2$ and 1U of Taq/Pfu mix (Taq and Pfu DNA polymerase were mixed in a ration of 9:1).

A typical amplification reaction comprised of;

1. Initial denaturation at 94°C for 5 minutes.
2. 30 cycles of denaturation at 94°C for 45 seconds, annealing at 55°C - 65°C for 1 minute and extension at 72°C for 1 minute.
3. Final extension at 72°C for 10 minutes.

The PCR products were resolved on 1.2 % agarose gel and purified by using Qiagen gel extraction kit, as described above.

Example 5: Transformation of *E. coli*:

E. coli XL-1 Blue and *E. coli* HB101 strains were grown in LB medium and competent cells were prepared by using the $CaCl_2$ method (Sambrook *et al.*, 1989). For preparation of high efficiency transformation cells, *E. coli* strains were grown to an A_{600nm} of 0.4 - 0.6 at 30°C and chilled at 4°C for 2 hours. The cells were harvested by pelleting the culture at 6,000 rpm at 4°C for 15 minutes. The cell pellet was resuspended in ice-cold trituration buffer (1/20th of the original culture volume) and diluted to the original culture volume by using prechilled tituration buffer. After incubating on ice for 45 minutes, cells were harvested by centrifugation at 6,000 rpm for 10 minutes at 4°C. The cell pellet was gently resuspended on ice-cold trituration buffer (1/10th of the original volume). Glycerol was added drop wise with gentle swirling to a final concentration of 15% (v/v) and competent cells were stored in aliquots of 1ml each at –70°C, till further use.

Transformation was carried out by the method described by Mandel and Higa (Mandel and Higa, 1970). The ligations or supercoiled DNA were mixed with 200 µl of cells and incubated on ice for 30 minutes. Cells were then subjected to heat shock at 42°C for 45 seconds, followed by incubation on ice for 2 minutes. After incubating on ice, 800 µl of LB medium was added to the cells and the sample was incubated at 37°C for one hr with constant shaking at 200rpm. The transformants were selected on LB agar plates supplemented with the appropriate antibiotic(s).

Example 6: Preparation of plasmid DNA from *E. coli* transformants This was carried out as per following protocols separately:

A) Mini-preparation of Plasmid DNA

(i) **By alkaline lysis - method:**

A single colony was inoculated in 3 ml of 2XYT medium containing appropriate antibiotic(s) and grown overnight at 37°C with shaking at 200 rpm. The cells were harvested by centrifugation at 6,000 rpm for 2 minutes at 4°C. The cell pellet was resuspended in 200 µl of TEG solution containing lysozyme (to a final concentration of 20 µg/ml) and the suspension was incubated at room temperature for 10 minutes. After incubating for 10 minutes 400 µl of freshly prepared alkaline - SDS solution was added followed by mixing and gentle inversion. After incubating on ice for 5 minutes, 300 µl of 3M potassium acetate was added, mixed by inversion and further incubated on ice for 10 minutes. The cell lysate was subjected to centrifugation at 12,000 rpm for 15 minutes at 4°C, followed by phenol chloroform extraction, followed by chloroform extraction, precipitated by adding 540 µl of isopropanol (0.6v/v) and DNA followed by centrifugation at 12,000 rpm for 10 minutes at room temperature. The pellet was washed twice with chilled 70% ethanol, air-dried and resuspended in 50 µl of TE buffer.

(ii) **By boiling lysis method:**

The bacterial culture was grown and harvested as described above. The cell pellet was resuspended in 600 µl of STET solution containing lysozyme (to a final concentration of 20 µg/ml). After incubating for 15 minutes at room temperature, the cell suspension was boiled at 100°C for 2 minutes. The clarified cell lysate was prepared by subjecting the crude cell lysate to centrifugation at 12,000 rpm for 15 minutes at room temperature. The DNA was precipitated by adding 600 µl of ammonia mix solution and recovered by centrifugation at 12,000 rpm for 10 minutes at room temperature. The pellet was washed twice with chilled 70% ethanol, air-dried and resuspended in 50 µl of TE buffer.

(iii) **By Qiagen miniprep kit:**

The bacterial culture was grown and harvested as described above. The pellet was resuspended in 250 µl of buffer P1 and incubated at room temperature for 5 minutes. After incubating for 5 minutes, 250 µl of buffer P2 was added and mixed by gentle inversions. After incubating for 5 minutes, 350 µl of buffer N3 was added and incubated on ice for 5 minutes and the clarified cell lysate was prepared by centrifugation at 12,000 rpm at 4°C for 15 minutes. The supernatant was passed through the Qia column, followed by washing with 500 µl of buffer PB. The column was

then washed twice with 750 µl of buffer PE. The purified DNA was eluted in 100 µl of elution buffer.

Maxi preparation of DNA:

Plasmid DNA was isolated on a large scale by the alkaline SDS method (Sambrook *et al* 1989). A single colony was inoculated in 200 ml of 2XYT medium containing appropriate antibiotic(s) and grown overnight at 37°C with shaking at 200 rpm. The cells were harvested by centrifugation at 6,000 rpm for 15 minutes at 4°C. The cell pellet was resuspended in 4 ml of Solution I containing lysozyme (to a final concentration of 20 µg/ml). The sample was incubated on ice for 30 minutes. After incubating on ice for 30 minutes, 8 ml of freshly prepared Solution II was added and the sample was further incubated on ice for 15 minutes. Then, 6 ml of Solution III was added and incubated on ice for 10 minutes. The clarified cell lysate was prepared by centrifugation at 12,000 rpm for 15 minutes at 4°C. The DNA was precipitated from the cell lysate by addition of 10.8 ml of isopropanol (0.6v/v). After incubating at room temperature for 10 minutes, plasmid DNA was recovered by centrifugation at 12,000 rpm for 15 minutes at room temperature. The pellet was washed twice with chilled 70% ethanol, air-dried and resuspended in 750 µl of TE buffer. The DNA was incubated with RNAaseA (20 µg/ml) for 30 minutes at 37°C, followed by extraction with phenol chloroform. DNA in the aqueous phase was precipitated by addition of 2.5 volumes of chilled absolute ethanol and sodium acetate to a final concentration of 0.3M. The DNA was incubated at –70°C for 15 minutes, and DNA was recovered by centrifugation at 12,000 rpm for 15 minutes at 4°C. The pellet was washed twice with 70% ethanol, air-dried and resuspended in 100 µl of TE buffer.

Example 7: DNA manipulations for Cloning Purposes:

Restriction Digestion of DNA:

The restriction enzyme digestions of DNA were carried out at the specified temperature, as per manufacturer's recommendations. The analytical digestion was carried out in a reaction volume of 20 µl and preparative digestions were carried out in a reaction volume of 100 µl.

Dephosphorylation of DNA termini:

Removal of 5' phosphate groups from DNA fragments was carried out by using Calf intestinal phosphatase. The DNA was incubated with the enzyme (1U) in 1X buffer at 37°C for 30 minutes followed by incubation at 56°C for 30 minutes. The enzyme was inactivated by incubating the reaction mixture at 65°C for 10 minutes

followed by phenol chloroform extraction and DNA was ethanol precipitated and resuspended in 10 µl of autoclaved double distilled water.

End filling of 5' overhang of DNA fragment:

DNA fragment with 5' overhang was end repaired by using Klenow fragment of DNA polymerase-I. The DNA (50ng/µl) was incubated with the enzyme (1-2U per µg of DNA) in 1X buffer containing 200 µM of dNTPs and incubated at 25°C for 15 minutes, followed by heat inactivation at 75°C for 15 minutes.

Ligation of DNA termini:

All the ligation reactions were carried out in a volume of 10 µl at 25°C for 3-4 hours. Each reaction contained typically 100 ng of the digested vector DNA; insert DNA fragment at 1:3 and 1:5 (vector: insert) molar concentrations and 1x ligase buffer containing 1mM ATP and 40U of T4 DNA ligase. The ligation mixtures were then used to transform competent cells of *E. coli* XL1-Blue and transformants were selected on appropriate LB agar supplemented with appropriate antibiotic(s).

Example 8: Agarose Gel Electrophoresis:

Agarose gel electrophoresis was carried out essentially as described earlier (Sambrook *et al.*, 1989). DNA fragments of size > 500 bp were resolved on 0.8% agarose gel, while those in the range of 250 - 500 bp were resolved on 1.2% agarose gel. The gels were electrophoresed in 1X TAE buffer containing 0.5 µg/ml ethidium bromide.

Example 9: Elution of DNA from Agarose:

DNA was eluted from agarose gel by using the Qiagen gel extraction kit. The gel was excised out and incubated with 3 gel volumes of QG buffer, at 55°C till the agarose was melted. The samples were then passed through Qia column, column was washed twice with PE buffer and the DNA was eluted in 50 µl of elution buffer.

Example 10 : Immunoblot analysis:

Protein samples were resolved on 10% SDS - PAGE and then transferred to Hybond C extra membrane overnight at 40mA or at 180mA for 2hours by using the Bio-Rad mini Trans Blot Cell (Bio-Rad Laboratories, Hercules, CA, USA). Transfer of the protein to the membrane was confirmed by staining with Ponceau S stain. The membrane was blocked in 2% milk for 2 hours at room temperature. The blot was than incubated with 1:10,000 dilution of the polyclonal sera for 2 hours at room temperature. To prevent non-specific binding of antibody, the dilutions were prepared in 2% milk-PBST. The blot was then washed thrice with PBST. After washing, the blot was

incubated with peroxidase conjugated goat anti-rabbit Immunoglobulin-G at a dilution of 1:2500. After incubation for 1 hour, the blot was washed thrice with PBST and the immunoreactive bands were visualized by the addition of PBS containing 10 µl/ml of 30% H_2O_2 and 0.5 mg/ml 3,3' diaminobenzidine tetrahydrochloride.

Example11: DNA sequencing

The DNA samples for sequencing were prepared from 3ml culture of the respective transformants using the Qiagen prep spin plasmid kit. The DNA samples were sequenced by using an ABI Prism 377 sequencer with rhodamine dye terminator chemistry.

The sequencing PCR reaction was set up in a PE-2400 thermocycler (Perkin Elmer – Cetus, Norwalk, Connecticut, USA) by using 500 ng double stranded DNA and 3.2 pmol vector specific oligonucleotides. After completion of the sequencing reactions, the extension products were precipitated with sodium acetate and ethanol to remove un-incorporated terminators. The samples were than loaded onto a 4% long ranger gel. The sample lanes were analysed on a DNA sequencing analysis 3.0 software (ABI-Prism, Perkin Elmer Applied Biosystems, Foster City, CA, USA).

Example 12: Electroporation of *M. tuberculosis*:

M. tuberculosis cultures were grown to A_{600nm} of 0.8 with shaking at 200 rpm at 37°C. Before harvesting, the cells were chilled on ice for one hour. Cells were pelleted by centrifugation at 6,000 rpm at 4°C for 10 minutes, washed twice with chilled glycerol (10%), resuspended in 1 ml of chilled glycerol (10%) and stored in aliquots of 100 µl each at –70°C, till further use.

For electroporation, approximately 2 µg of DNA was mixed with 20 µl of cells, kept on ice for 15 minutes and cells were subsequently pulsed at field strength of 16 kV/cm (400 V input, 330µF capacitance, 8kOhms resistance, 2.4 kV output using cuvette with 0.15 cm gap width). Cells were recovered in 1ml of 7H9 medium at 37°C, 200 rpm for 24 hours. The transformants were selected on Middlebrook 7H10 agar plates supplemented with ADC and containing appropriate antibiotic(s). Plates were incubated for 14-21 days at 37°C.

Example13: Alkali and U.V. pretreatment of DNA:

The targeting DNA was pretreated with alkali before its electroporation into the competent cells of *M. tuberculosis* as per the method described by Hinds *et al* 1999 (Hinds *et al.*, 1999). The vector was denatured in 20 µl of 0.2M NaOH containing 0.2 mM EDTA for 30 minutes at 37°C. The denatured DNA was precipitated by addition of $1/10^{th}$ volume of 3M sodium acetate and 2.5 volumes of chilled absolute ethanol. The

DNA was precipitated by incubating the samples at –70°C for 15 minutes and recovered by centrifugation at 12,000 rpm for 15minutes at 4°C. The pellet was washed twice with chilled 70% ethanol to remove salts, air dried and resuspended in 10 µl of double distilled water. For U.V. pretreatment, DNA was subjected to U.V. irradiation in an U.V. stratalinker 1800 (Amersham) at 100-mJ cm^{-2} for 5 minutes. For alkali and U.V. pretreatment of DNA, the DNA was prepared by Qiagen column as described above.

Example 14: Southern Blot hybridization:

The genomic DNA was isolated from *M. tuberculosis*, and subjected to restriction digestion by appropriate restriction endonuclease. The digested fragments were resolved on a 1.2% agarose gel at low voltage (40V) overnight in 1X TAE gel running buffer. The DNA fragments were depurinated by soaking the gel in 0.1N HCl for 10 minutes followed by a wash with double distilled water. The DNA was then denatured by soaking the gel in denaturation buffer (1.5M NaCl, 0.5 M NaOH). The gel was then rinsed with double distilled water and neutralized in neutralization buffer (1M Tris pH 7.4, 1.5 M NaCl). The DNA was then transferred to Hybond N membrane by capillary transfer in 20X SSC overnight (Southern 1975). The membrane was air-dried and DNA was cross-linked to the Hybond N membrane by U.V. irradiation for 2 minutes at 700mJ. The blot was prehybridized in a solution containing 50% deionised formamide, 5X SSC, 5X Denhardts solution, 50mM Tris-Cl, pH7.5 and 200 µg/ml denatured salmon sperm DNA overnight at 42°C. The heat denatured probe was then added to the blots and hybridization was carried out at 42°C for 14-16 hours. The blot was washed first in 2X SSC and 0.1% SDS at room temperature for 30 minutes and then in 0.2X SSC and 0.1% SDS at room temperature for 30 minutes and then in 0.2X SSC and 0.2% SDS at 65°C for 30 minutes. The blot was then air dried, wrapped in saran wrap and subjected to autoradiography.

Example 15: Preparation of Nucleic Acid Probes:

The DNA fragment to be labeled was PCR amplified by using gene specific primers. The amplicon was purified by using Qiagen gel extraction kit and end-labeled by using NEBlot kit in a 50 µl reaction. The labeling reaction comprised of 100ng of template DNA, 1x klenow buffer (having random primers), 1mM dGTP, 1mM dCTP, 1mM dTTP and 10uCi of $\alpha^{32}P$ dATP, 1U of klenow fragment. The template DNA was denatured at 100°C for 5 minutes and kept in ice for 2 minutes, dNTPs and enzyme were added and end labeling was carried out at 37°C for 2hours. Unincorporated

dNTPs were removed by using Qiagen nucleotide removal kit and the labeled probe was added to the blot.

Example 16: *In vitro* infection of mouse macrophage cell line by *M. tuberculosis*:

J774A.1 mouse macrophage cell line (resting or activated with rIFN-γ 50Uml^{-1} for 16hours) was seeded in a six well plate at a density of 2x10^5 per well. Before infection, the cell lines were washed once with 1 x Hanks Balanced Salt Solution (HBSS) and medium was replaced with Dulbeccos modified eagle's medium (DMEM) supplemented with 10% heat inactivated fetal calf serum (FCS). The bacterial strains were washed twice with DMEM and resuspended in DMEM supplemented with 5% FCS. The cells were infected with wild type or mutant strain at an MOI of 1:10 (macrophage : bacteria). The cells were incubated at 37°C in a 5% CO_2 atmosphere. After 6 hours of infection, cells were washed twice with 1x HBSS and overlayed with 2 ml DMEM supplemented with FCS (10%), Antibiotic-antimycotic (1%) and amikacin (20 μg/ml). On days 0, 2, 4, 6 and 8, infected cells were lysed in 1 ml of 0.1% Triton X-100 for 15 minutes. The number of bacilli at different time points was determined by plating 10-fold serial dilutions in duplicates on MB 7H10 medium and incubating the plates at 37°C for 3 weeks.

Example 17: Virulence studies in guinea pigs:

The effect of disruption of tyrosine phosphatases on the virulence of *M. tuberculosis* was evaluated in the guinea pig model of experimental tuberculosis. This work was carried at Tuberculosis Research centre, Chennai. Random-Bred guinea pigs of the Duncan-Hartley strain in the weight range of 200 - 400g were obtained from National Center for Laboratory Animal Science (NCLAS), Hyderabad.

The guinea pigs were divided into groups of sixteen each. Each group comprised of 16 animals, 8 males and 8 females. The different groups of guinea pigs were challenged with one of the organisms mentioned below subcutaneously and 8 animals (4 males and 4 females) were euthanised at 3 weeks and 6 weeks post – challenge.

a) *M. tuberculosis* Erdman b) *mptpA* mutant strain	High Dose (5 x 10^7)
c) *M. tuberculosis* Erdman d) *mptpB* mutant strain	Low Dose (5 x 10^5)

e) *mptpB* complemented strain

All the organisms were coded and animals were subcutaneously challenged with all the coded preparations separately by using a 1ml tuberculin syringe with a 26 G needle.

After euthanasia the following investigations were carried out

1) Gross body weight of the animal.
2) Weight of infected organs – liver, spleen and lung.
3) Scores of the gross pathological damage to the organs (Post-mortem scores).
4) Viable count of the tubercle bacilli from spleen and lung (Bacterial enumeration).
5) Histopathological evaluation of liver and lung.

The gross body weight of the animals was measured at the time of beginning of the experiment, and at weekly intervals till euthanasia. Liver, lungs, spleens and lymph nodes were removed aseptically and the weight of the infected organs was measured. The bacterial load was enumerated in spleens and lungs. Portions of liver and lung tissues were fixed in 10% formalin for histopathological analysis of granuloma formation and cellular composition of granuloma.

***Post-mortem* scores:**

The virulence was measured based on the rate of progression of the disease in guinea pig as described by Mitichison. (Mitichison, 1964). At the post-mortem examination of the animals, the total extent of tuberculosis disease was assessed as a score ranging from 0 to 100. The extent of visible lesions in the organs were scored as described in Table 5. Average score for each group was calculated.

***Viable count* of the tubercle bacilli from the spleen and lung:**

The spleen and portion of lung was removed into a sterile, weighed grinding tube. Organs were homogenised in 5ml of double distilled water by using a teflon homogenizer. Ten fold serial dilutions (10^{-1}, 10^{-2},10^{-3},10^{-4}) were prepared in distilled water and 10µl of neat homogenate and various dilutions were inoculated in LJ slopes in duplicates. The LJ slopes were incubated at 37°C and readings for cfu were taken after 4 weeks and 6 weeks. The number of cfu per organ and an average organ cfu for each group was calculated. The sensitivity of this detection method was 500 bacilli.

Histopathology of liver and lungs:

The liver and lungs of the animals were removed and stored in preweighed jars containing 10% formaldehyde. Two bits of tissue (2cm x 2cm thickness) each from liver and lung were fixed in 10% formalin until further treatment. The organ bits were washed in 70% alcohol and 95% alcohol for 2 hours each followed by treatment with isopropanol for 2 hours. In order to ensure complete dehydration of the tissue, the isopropanol treatment was repeated twice. The bits were then incubated in xylene for 15-20 minutes and finally embedded in molten paraffin wax. The paraffin embedded tissue portions was divided into 5 μm fine sections by using a microtome (Reichert, Germany) and fixed onto glass slides. Deparaffinization of the cut sections was carried out prior to staining. The slides were first immersed twice in xylene for 5 minutes each followed by treatment with isopropanol twice for 3 minutes each. The slides were finally treated with 95% alcohol for complete removal of traces of wax.

The sections were stained with hematoxylin and eosin for the presence of granuloma. The sections were washed in water and stained with hematoxylin for 5 minutes. Excess stain from the slide was removed by washing with distilled water. The slides were then counterstained with eosin solution for 1 minutes, washed with water and air-dried. For viewing the slides under the microscope, the slides were mounted using DPS mount and covered with a coverslip. The proportion of the granuloma and extent and type of cellular infiltration in the sections were microscopically assessed as described earlier (Ridley, 1977 and Jayashankar and Ramanathan, 1999). The tissue sections were analysed for following parameters to determine the effect of disruption of tyrosine phosphatases on the virulence of *M. tuberculosis*; size of typical granuloma; amount of caseous necrosis; relative number of neutrophils; macrophages; giant cells; epitheloid cells and lymphocytes; degree to which lymphocytes were organized in the granuloma and extent to which granuloma were organized. At least four different sections for each tissue were analyzed.

Statistical analysis:

Data are depicted as arithmetic mean $\pm$ standard error mean. Data were analyzed for statistical significance using the Student's t test. Differences between the various groups of guinea pig were considered significant if p values were <0.05.

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University of Delhi

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We claim:

1) A *Mycobacterium* strain with a modified tyrosine phosphatase gene in its genome, wherein the said *Mycobacterium* strain is incapable of expressing the active tyrosine phosphatase gene.
2) The *Mycobacterium* strain as claimed in claim 1 wherein the *Mycobacterium* species is selected from a group consisting of *M. tuberculosis* and *M. bovis*.
3) The *Mycobacterium* strain as claimed in claim 1 wherein the modified tyrosine phosphatase gene is modified *mptpA* gene.
4) The *Mycobacterium* strain as claimed in claim 3 wherein the modified *mptpA* gene is as shown in SEQ ID NO : 15.
5) The *Mycobacterium* strain as claimed in claim 1 wherein the modified tyrosine phosphatase gene is modified *mptpB* gene.
6) The *Mycobacterium* strain as claimed in claim 5 wherein the modified *mptpB* gene is as shown in SEQ ID NO : 16.
7) A recombinant vector comprising the modified *mptpA* gene of claim 3.
8) A recombinant vector as claimed in claim 7 is pAKΔA.
9) A recombinant vector comprising the modified *mptpB* gene of claim 5.
10) A recombinant vector as claimed in claim 9 is pBKΔB.
11) The recombinant vector as claimed in claim 7, wherein the nucleotide sequence of *mptpA* gene as shown in **SEQ ID NO: 11** is modified.
12) The recombinant vector as claimed in claim 9, wherein the nucleotide sequence of *mptpB* gene as shown in **SEQ ID NO: 12** is modified.
13) The recombinant vector as claimed in claim 7 or 9, wherein the *mptpA* or *mptpB* gene is modified by insertion, deletion, mutation or substitution.
14) The recombinant vector as claimed in claim 7 or 9, wherein the *mptpA* or *mptpB* gene is modified by substituting an internal region of the *mptpA or mptpB* gene by an antibiotic resistance marker gene.
15) The recombinant vector as claimed in claim 14, wherein the antibiotic resistance marker gene imparts resistance to either hygromycin or chloramphenicol preferably to hygromycin.
16) The recombinant vector as claimed in claim 7 or 9, wherein a second antibiotic marker gene is inserted in the backbone of the said recombinant vector.
17) The recombinant vector as claimed in claim 16, wherein the second antibiotic marker gene imparts resistance to kanamycin or gentamycin.

18) An isolated nucleotide sequence of the *mptpA* gene encoding the mycobacterial tyrosine phosphatase A as shown in SEQ ID NO : 11.

19) An isolated nucleotide sequence of the *mptpB* gene encoding the mycobacterial tyrosine phosphatase B as shown in SEQ ID NO : 12.

20) An isolated nucleotide sequence of the modified *mptpA* gene as shown in SEQ ID NO : 15.

21) An isolated nucleotide sequence of the modified *mptpB* gene as shown in SEQ ID NO : 16.

22) A method for developing a *Mycobacterium* strain with a modified tyrosine phosphatase gene in its genome comprising the following steps:

a. extracting genomic DNA from *Mycobacterium* strain,

b. amplifying the tyrosine phosphatase gene along with the flanking sequences using specific primers from the genomic DNA of step (a) to obtain a DNA fragment,

c. characterizing the fragment of step (b),

d. cloning the fragment of step (b) in a non-replicative vector,

e. modifying the fragment in the non-replicative vector of step (d),

f. inserting an antibiotic resistance marker gene within the fragment of step (e) to obtain a non-replicative vector containing a modified tyrosine phosphatase gene,

g. cloning of a second antibiotic resistance marker gene in the backbone of the non-replicative vector of step (f), to obtain a recombinant vector,

h. introducing the recombinant vector of step (g) into *Mycobacterium strains*,

i. selecting for primary recombinant *Mycobacterium strains* using the first antibiotic selection marker gene,

j. culturing the primary recombinant *Mycobacterium strains* of step (i) harboring the first antibiotic resistance marker gene,

k. selecting the secondary recombinant *Mycobacterium strains* of step (j) that is sensitive to the second antibiotic resistance gene present in the vector backbone,

l. culturing the secondary recombinant *Mycobacterium strains* of step (k), wherein the said recombinant *Mycobacterium* strain harboring the modified tyrosine phosphatase gene which shows defective growth in activated macrophages and animals.

23) The method as claimed in claim 22, wherein the *Mycobacterium* species is selected from a group consisting of *M. tuberculosis* and *M. bovis*.

24) The method as claimed in claim 22, wherein in step (b) the specific primers are selected from a group comprising of SEQ ID NO : 1 to 4 for amplification of *mptpA* along with its flanking regions and SEQ ID NO : 5 to 8 for amplification of *mptpB* along with its flanking regions.

25) The method as claimed in claim 22, wherein in step (b) the tyrosine phosphatase gene is *mptpA* gene as shown in SEQ ID NO : 11.

26) The method as claimed in claim 22, wherein in step (b) the tyrosine phosphatase gene is *mptpB* gene as shown in SEQ ID NO : 12.

27) The method as claimed in claim 22, wherein in step (b) the DNA fragment is a sequence as shown in SEQ ID NO : 13.

28) The method as claimed in claim 22, wherein in step (b) the DNA fragment is a sequence as shown in SEQ ID NO : 14.

29) The method as claimed in claim 22, wherein in step (c) the DNA fragment is characterized by sequencing and restriction enzyme analysis.

30) The method as claimed in claim 22, wherein in step (f) the modified tyrosine phosphatase gene is modified *mptpA* gene as shown in SEQ ID NO : 15.

31) The method as claimed in claim 22, wherein in step (f) the modified tyrosine phosphatase gene is modified *mptpB* gene as shown in SEQ ID NO : 16.

32) The method as claimed in claim 30 or 31, wherein the *mptpA* or *mptpB* gene is modified by insertion, deletion, mutation or substitution.

33) The method as claimed in claim 30 or 31, wherein the *mptpA* or *mptpB* gene is modified by substituting an internal region of the *mptpA or mptpB* gene by an antibiotic resistance marker gene.

34) The method as claimed in claim 33, wherein the antibiotic resistance marker gene imparts resistance to either hygromycin or chloramphenicol preferably to hygromycin.

35) The method as claimed in claim 22, wherein in step (g) the second antibiotic marker gene imparts resistance to kanamycin.

36) The method as claimed in claim 22, wherein in step (g) the recombinant vector is either pAKΔA or pBKΔB.

37) The method as claimed in claim 22, wherein in step (h) the introduction of the vector is by either electroporation or phages.

38) The method as claimed in claim 22, wherein in step (i) the selection of primary recombinant *Mycobacterium* strain is by using either hygromycin or chloramphenicol.

39) The method as claimed in claim 22, wherein in step (k) the selection of secondary recombinant *Mycobacterium* strain which are resistant to either hygromycin or chloramphenicol but sensitive to second antibiotic resistance marker (kanamycin).

40) A *Mycobacterium* strain with a modified tyrosine phosphatase gene in its genome, substantially as herein described with reference to the accompanying drawings.

41) A recombinant vector, substantially as herein described with reference to the accompanying drawings.

42) An isolated nucleotide sequence of the *mptpA* gene, substantially as herein described with reference to the accompanying drawings.

43) An isolated nucleotide sequence of the *mptpB* gene, substantially as herein described with reference to the accompanying drawings.

44) A method for developing a *Mycobacterium* strain with a modified tyrosine phosphatase gene, substantially as herein described with reference to the accompanying drawings.

Dated this 9th day of July, 2004

Rajeshwari

RAJESHWARI H.
OF K & S PARTNERS
ATTORNEY FOR THE APPLICANT

Abstract

The present invention provides mutant *Mycobacterium* strains harboring a modified tyrosine phosphatase gene (*mptpA* or *mptpB*) wherein the mutant *Mycobacterium* strain is incapable of expressing the active tyrosine phosphatase. The invention provides a method for developing the said mutant strain from either *Mycobacterium tuberculosis* or *Mycobacterium bovis*. The *mptpA* or *mptpB* gene may be modified by replacing the internal sequences with an antibiotic resistance marker gene, which disrupts the expression of the active gene. The invention further provides a recombinant vector comprising the modified *mptpA* or *mptpB* which may be used to develop the mutant strains of mycobacteria. The invention provides a method to assess the role of tyrosine phosphatases MptpA and MptpB in the virulence and pathogenesis of *Mycobacterium* which can be used as potential targets for developing anti-tubercular drug.

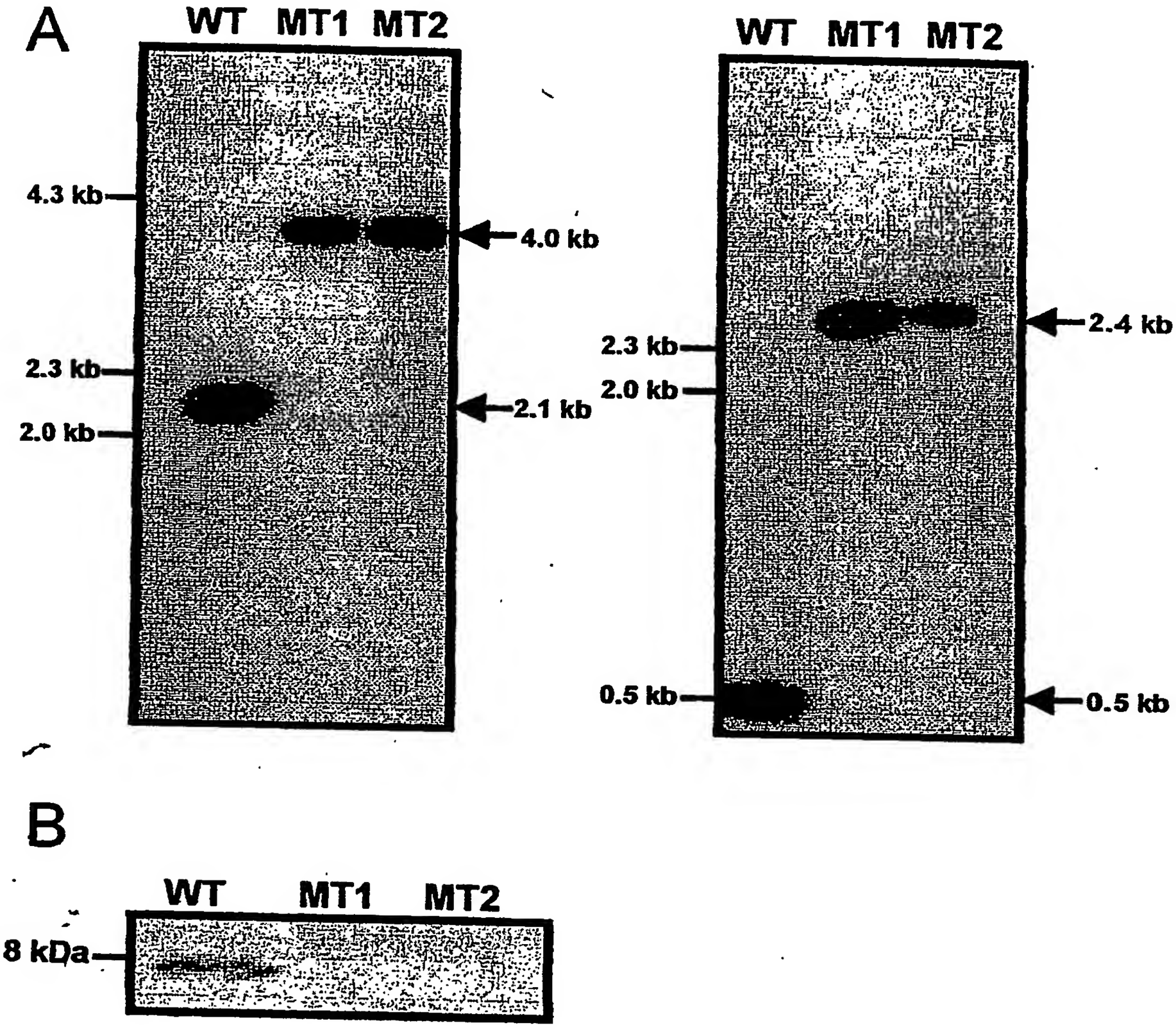

Fig. 1

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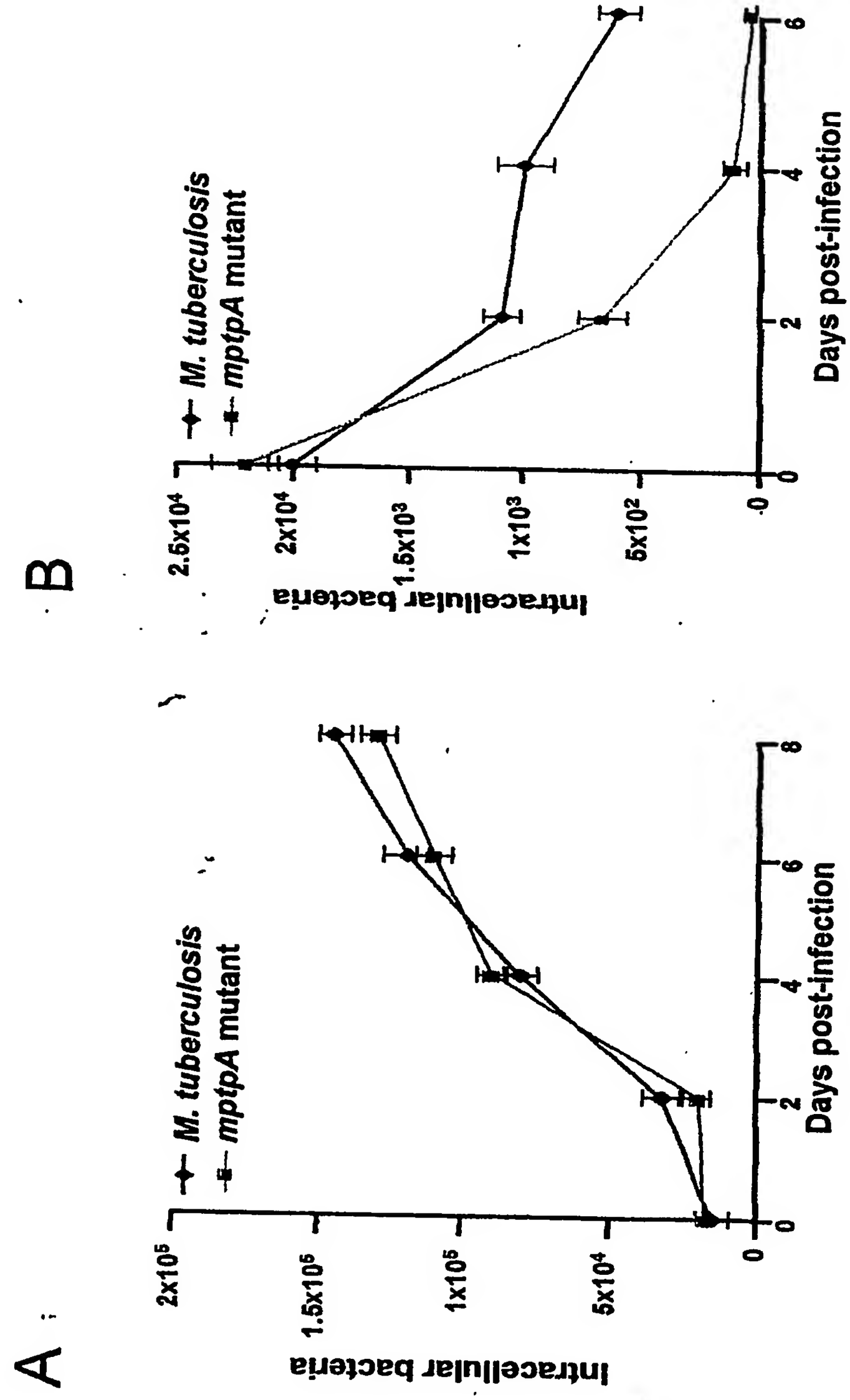

Fig. 2

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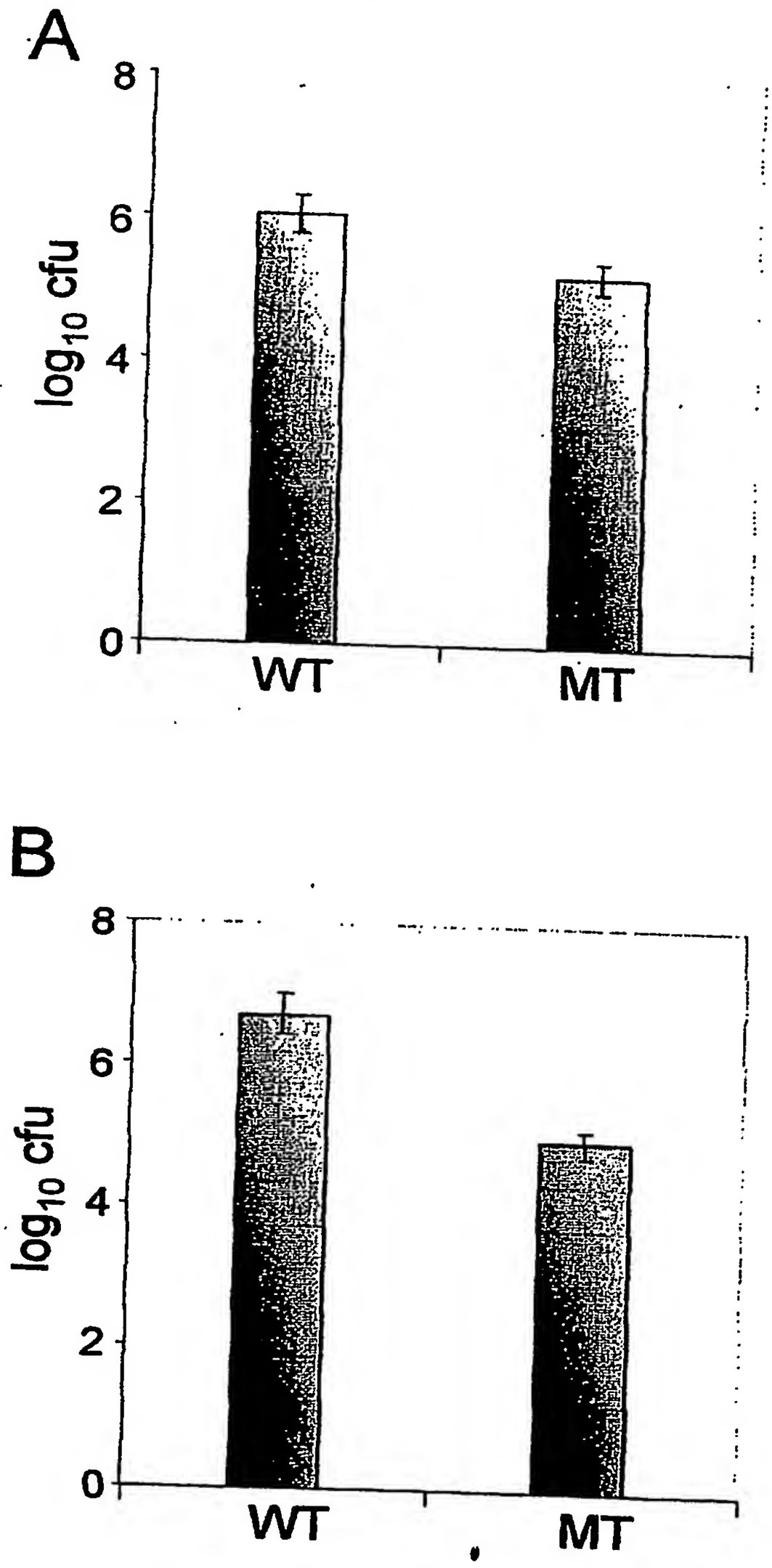

DUPLICATE

Fig. 3

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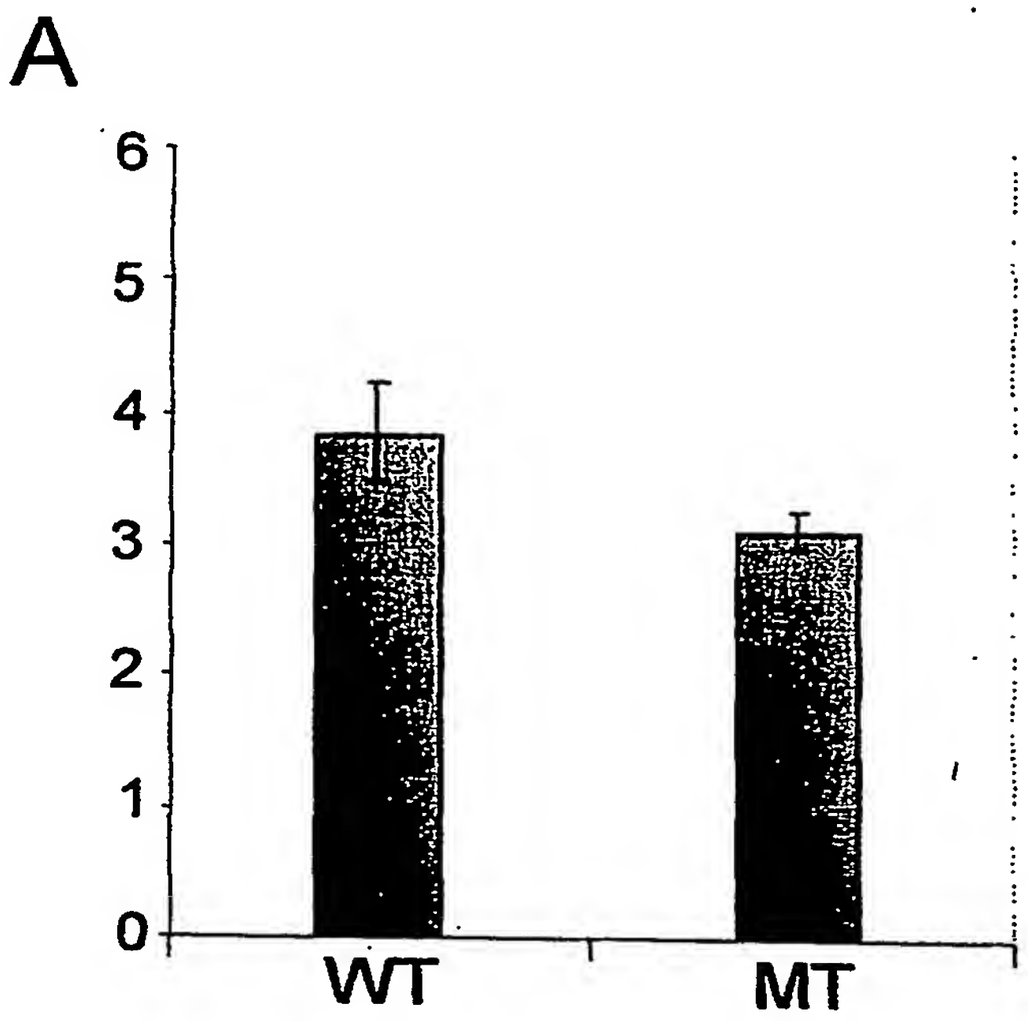

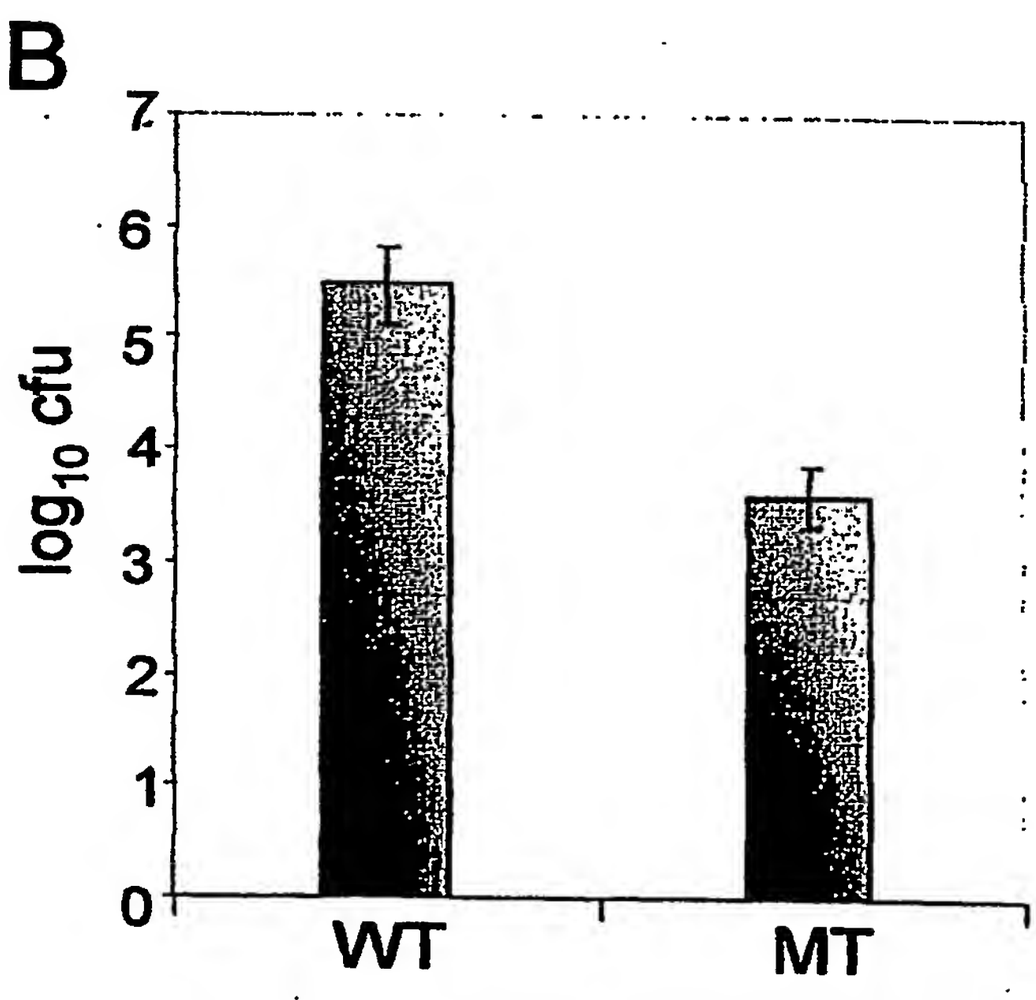

Fig. 4

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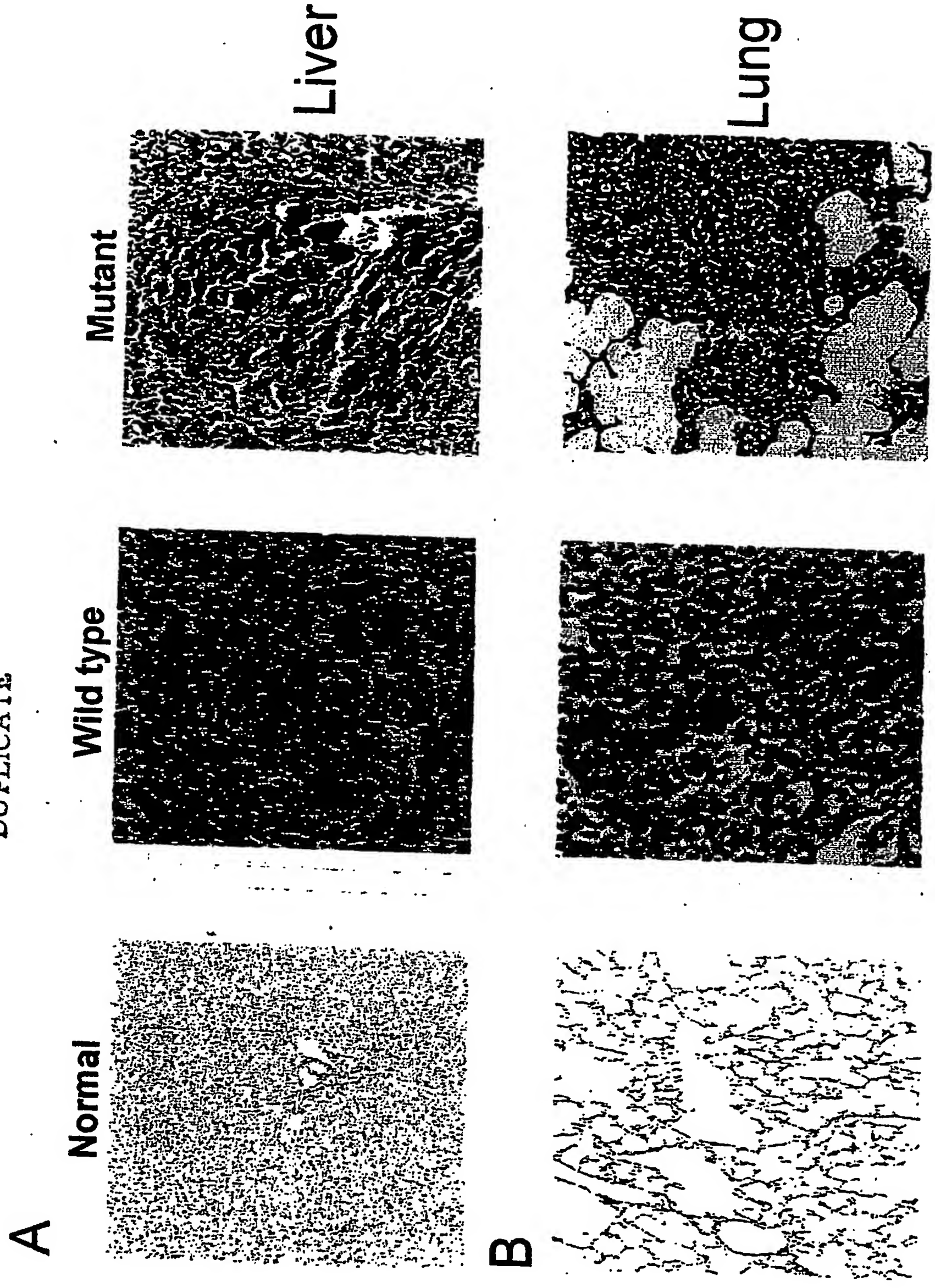

Fig. 5

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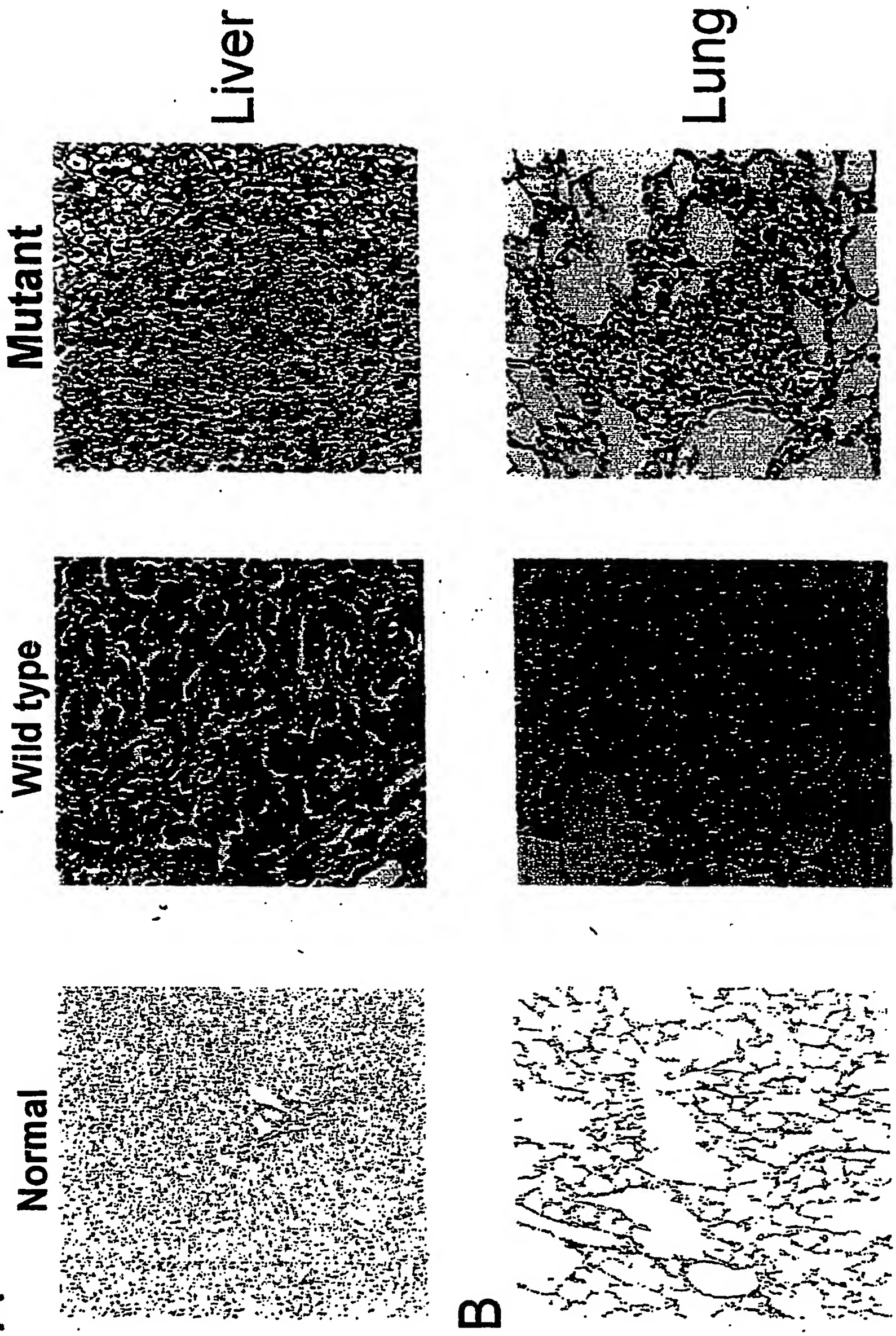

Fig. 6

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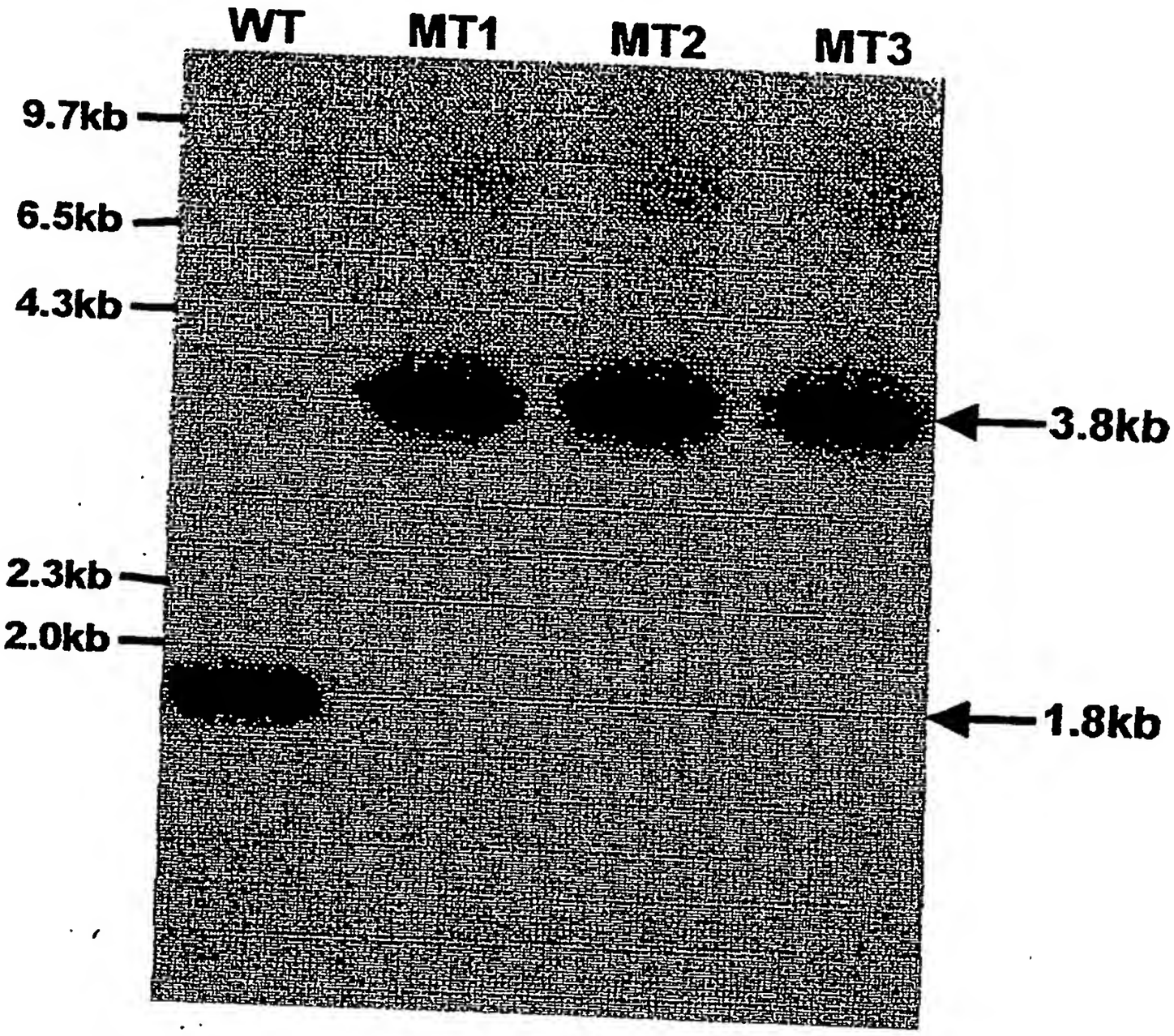

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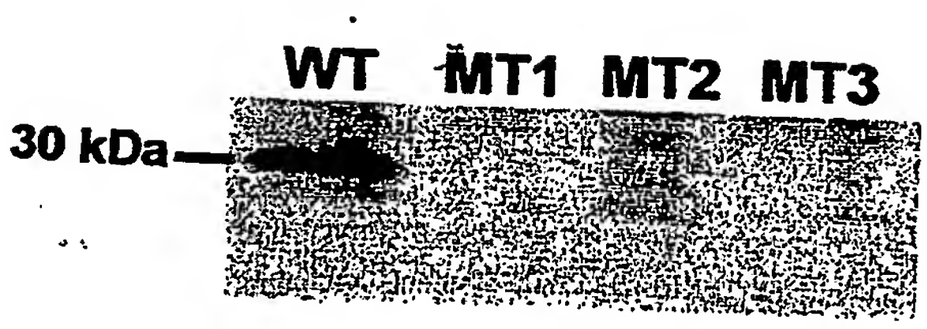

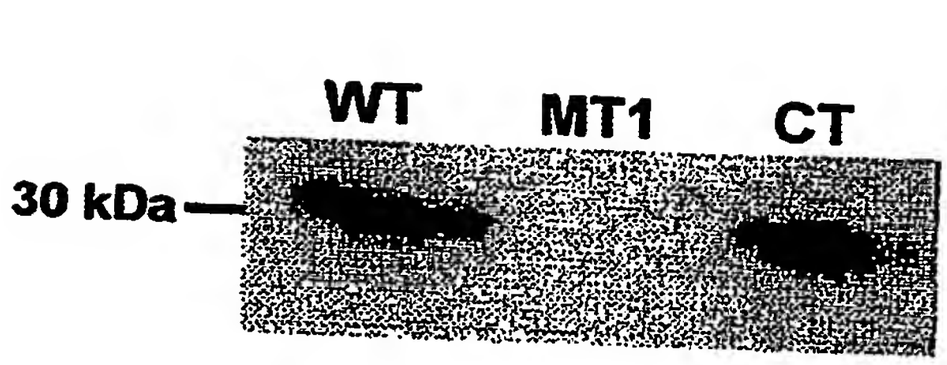

Fig. 7

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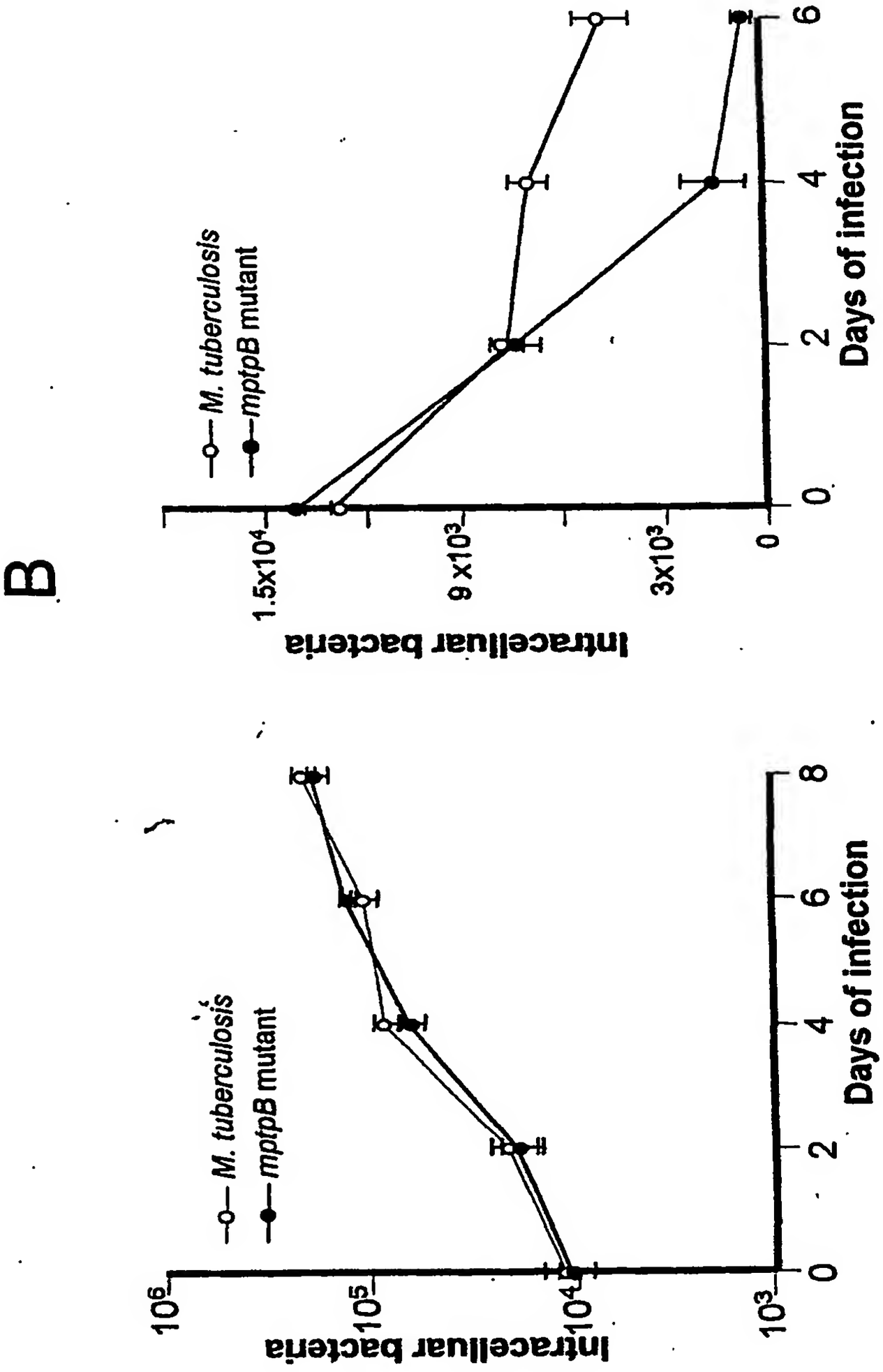

Fig. 8

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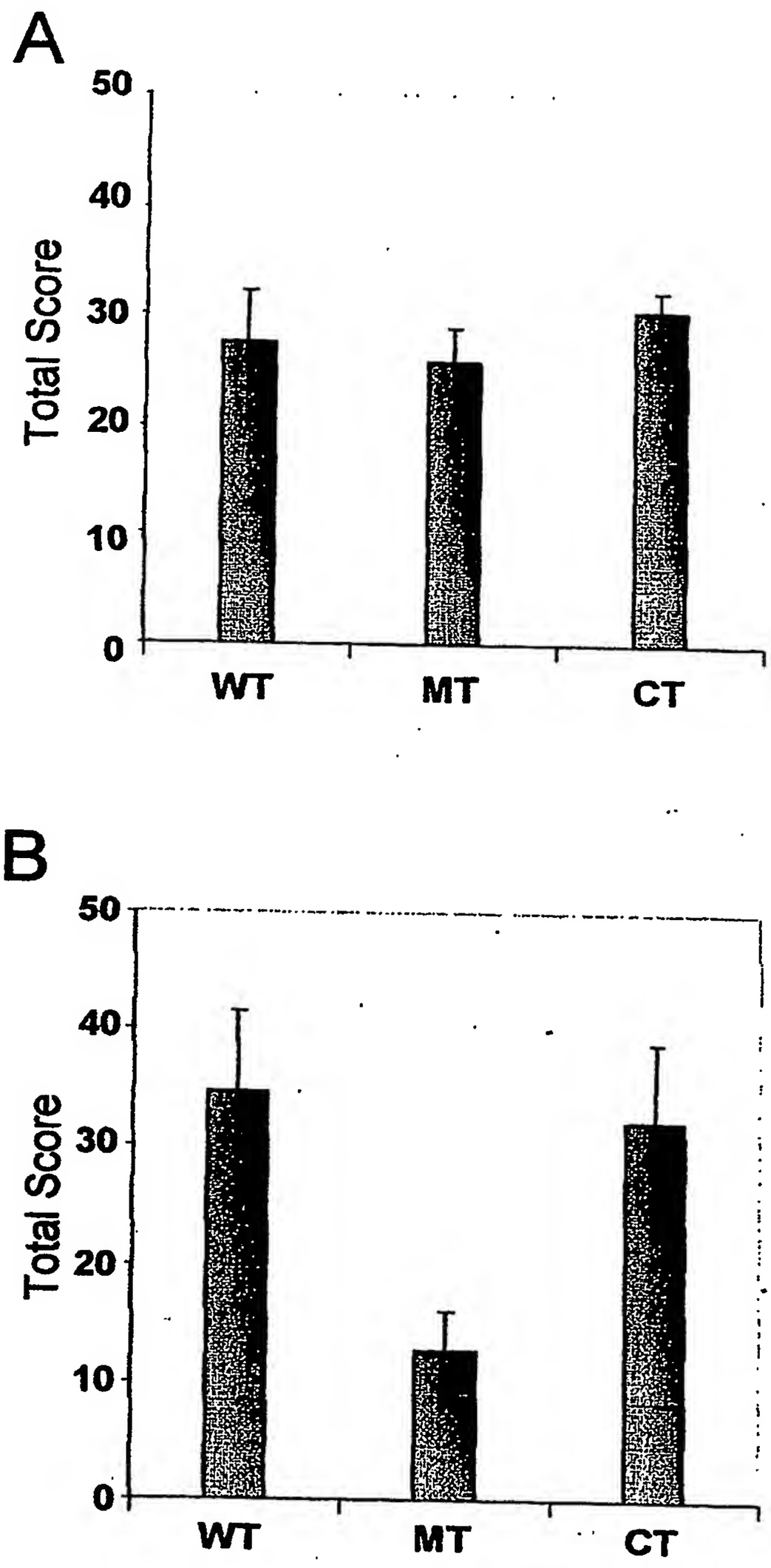

Fig. 9

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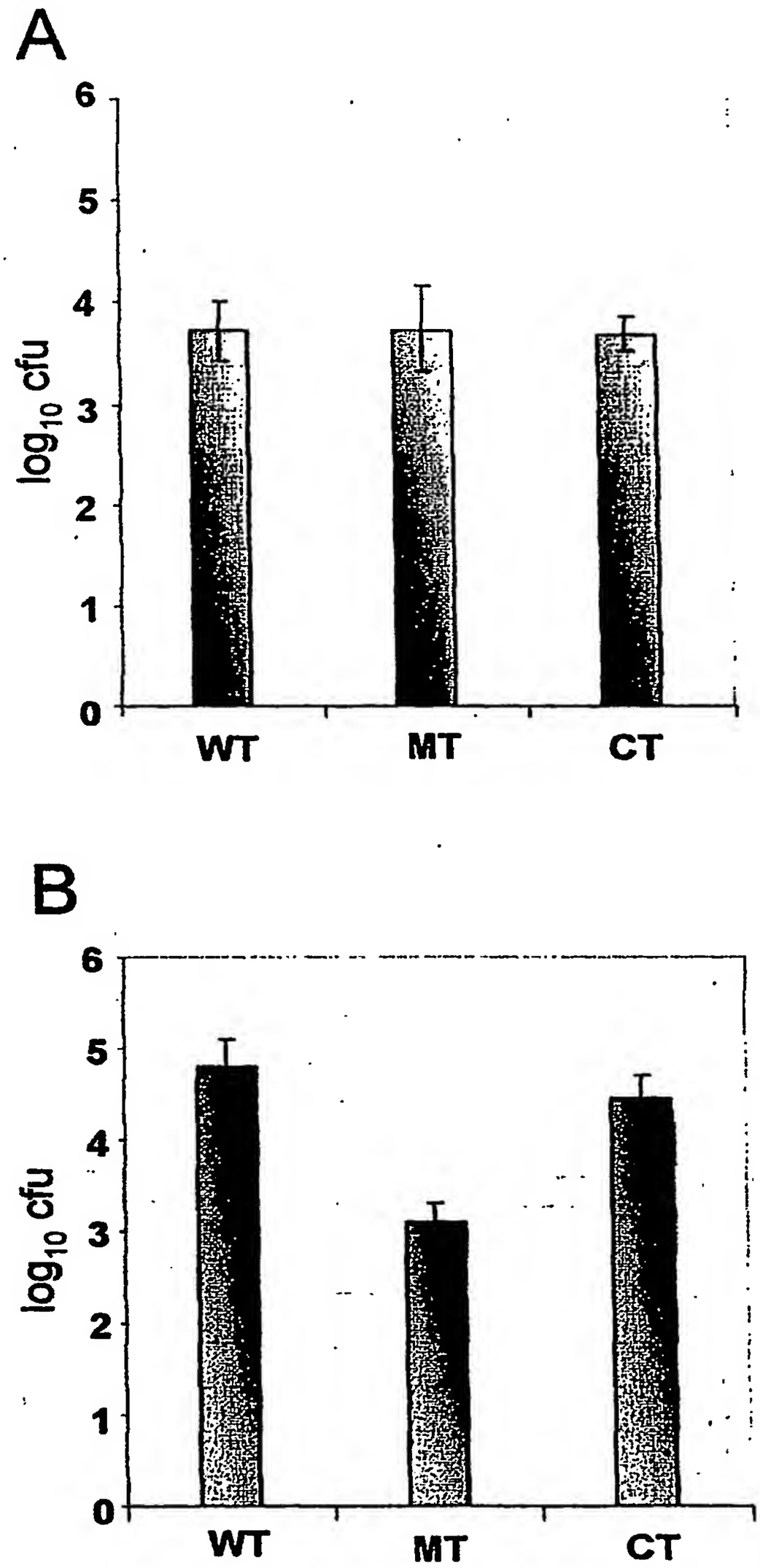

Fig. 10

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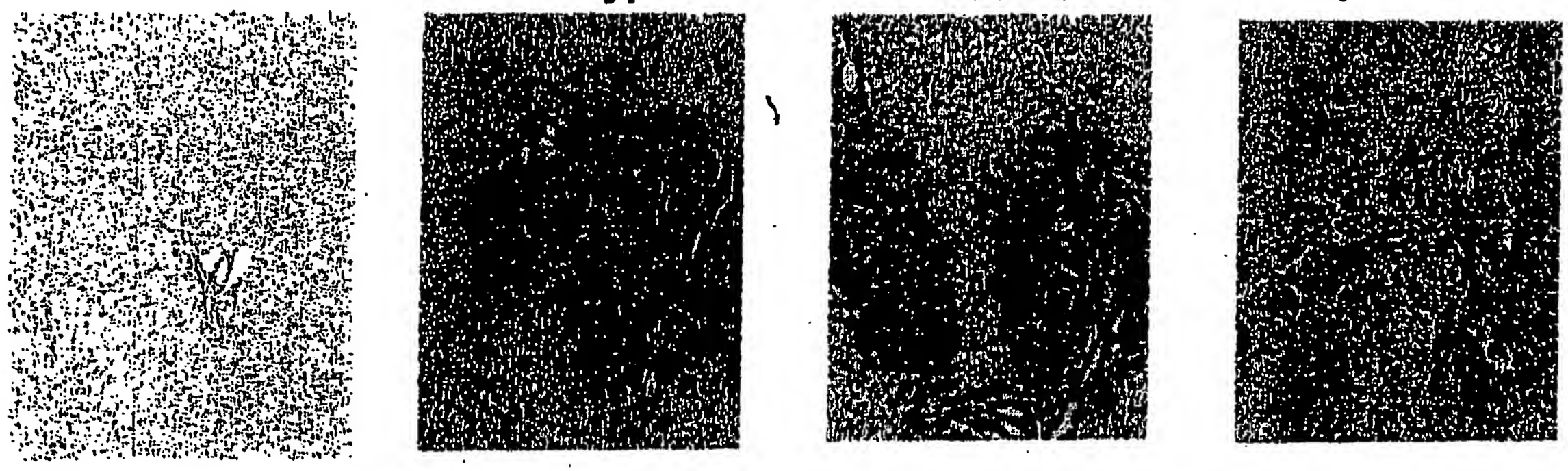

Fig. 11

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and UNIVERSITY OF DELHI
INDIAN APPL. NO.882/DEL/2003 dt. 09.07.2003

TOTAL SHEET: 12
SHEET #: 11

- 9 JUL 2003

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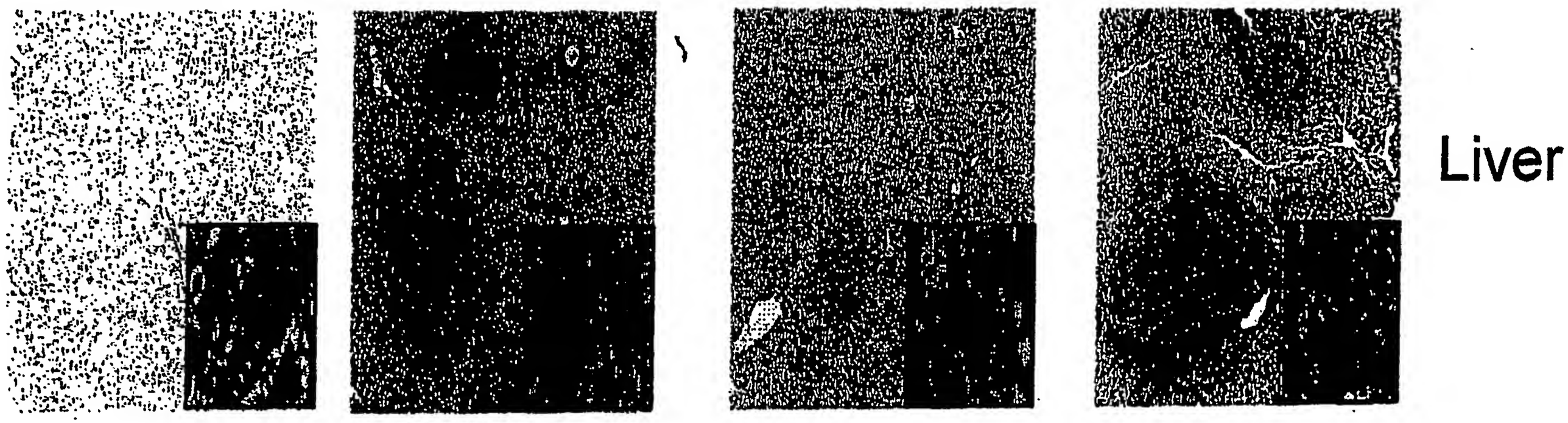

Fig. 12

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